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THE

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(On File in the Office of the Secretary of the Commonwealth)

No. 3170

We. Alpheus Hyatt, President, William Stanford Stevens, Treasurer, and William T. Sedgwick, Edward G. Gardiner, Susan Mims and Charles Sedgwick Minot being a majority of the Trustees of the Marine Biological Laboratory in compliance with the requirements of the fourth section of chapter one hundred and fifteen of the Public Statutes do hereby certify that the following is a true copy of the agreement of association to constitute said Corporation, with the names of the subscribers thereto:

We, whose names are hereto subscribed, do, by this agreement, associate ourselves with the intention to constitute a Corporation according to the provisions of the one hundred and fifteenth chapter of the Public Statutes of the Commonwealth of Massachusetts, and the Acts in amendment thereof and in addition thereto.

The name by which the Corporation shall be known is THE MARINE BIOLOGICAL LABORATORY.

The purpose for which the Corporation is constituted is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

The place within which the Corporation is established or located is the city of Boston within said Commonwealth.

The amount of its capital stock is none.

In Witness Whereof, we have hereunto set our hands, this twenty seventh day of February in the year eighteen hundred and eighty-eight, Alpheus Hyatt, Samuel Mills, William T. Sedgwick, Edward G. Gardiner, Charles Sedgwick Minot, William G. Farlow, William Stanford Stevens, Anna D. Phillips, Susan Mims, B. H. Van Vleck.

That the first meeting of the subscribers to said agreement was held on the thirteenth day of March in the year eighteen hundred and eighty-eight.

In Witness Whereof, we have hereunto signed our names, this thirteenth day of March in the year eighteen hundred and eighty-eight, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, Edward G. Gardiner, William T. Sedgwick, Susan Mims, Charles Sedgwick Minot.

(Approved on March 20, 1888 as follows:

I hereby certify that it appears upon an examination of the within written certificate and the records of the corporation duly submitted to my inspection, that the requirements of sections one, two and three of chapter one hundred and fifteen, and sections eighteen, twenty and twenty-one of chapter one hundred and six, of the Public Statutes, have been complied with and I hereby approve said certificate this twentieth day of March A.D. eighteen hundred and eighty-eight.

CHARLES ENDICOTT
Commissioner of Corporations)

IV. ARTICLES OF AMENDMENT

(On File in the Office of the Secretary of the Commonwealth)

We. James D. Ebert, President, and David Shepro, Clerk of the Marine Biological Laboratory, located at Woods Hole, Massachusetts 02543, do hereby certify that the following amendment to the Articles of Organization of the Corporation was duly adopted at a meeting held on August 15, 1975, as adjourned to August 29, 1975, by vote of 444 members, being at least two-thirds of its members legally qualified to vote in the meeting of the corporation:

VOTED: That the Certificate of Organization of this corporation be and it hereby is amended by the addition of the following provisions:

> "No Officer, Trustee or Corporate Member of the corporation shall be personally liable for the payment or satisfaction of any obligation or liabilities incurred as a result of, or otherwise in connection with, any commitments, agreements, activities or affairs of the corporation.
>
> "Except as otherwise specifically provided by the Bylaws of the corporation, meetings of the Corporate Members of the corporation may be held anywhere in the United States.
>
> "The Trustees of the corporation may make, amend or repeal the Bylaws of the corporation in whole or in part, except with respect to any provisions thereof which shall by law, this Certificate or the bylaws of the corporation, require action by the Corporate Members."

The foregoing amendment will become effective when these articles of amendment are filed in accordance with Chapter 180, Section 7 of the General Laws unless these articles specify, in accordance with the vote adopting the amendment, a later effective date not more than thirty days after such filing, in which event the amendment will become effective on such later date.

In Witness whereof and Under the Penalties of Perjury, we have hereto signed our names this 2nd day of September, in the year 1975, James D. Ebert, President; David Shepro, Clerk.

(Approved on October 24, 1975, as follows:

I hereby approve the within articles of amendment and, the filing fee in the amount of $10 having been paid, said articles are deemed to have been filed with me this 24th day of October, 1975.

PAUL GUZZI
Secretary of the Commonwealth)

V. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 16, 1985)

I. (A) The name of the Corporation shall be The Marine Biological Laboratory. The Corporation's purpose shall be to establish and maintain a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history.

(B) Marine Biological Laboratory admits students without regard to race, color, sex, national and ethnic origin to all the rights, privileges, programs and activities generally accorded or made available to students in its courses. It does not discriminate on the basis of race, color, sex, national and ethnic origin in employment, administration of its educational policies, admissions policies, scholarship and other programs.

II. (A) The members of the Corporation ("Members") shall consist of persons elected by the Board of Trustees, upon such terms and conditions and in accordance with such procedures, not inconsistent with law or these Bylaws, as may be determined by said Board of Trustees. Except as provided below, any Member may vote at any meeting either in person or by proxy executed no more than six months prior to the date of such meeting. Members shall serve until their death or resignation unless earlier removed with or without cause by the affirmative vote of two-thirds of the Trustees then in office. Any member who has attained the age of seventy years or has retired from his home institution shall automatically be designated a Life Member provided he signifies his wish to retain his membership. Life Members shall not have the right to vote and shall not be assessed for dues.

(B) The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

III. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk, elected or appointed by the Trustees as set forth in Article IX.

IV. The Annual Meeting of the Members shall be held on the Friday following the Second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 a.m. Subject to the provisions of Article VIII(2), at such meeting the Members shall choose by ballot six Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the Members may be called by the Chairman or Trustees to be held at such time and place as may be designated.

V. Twenty five Members shall constitute a quorum at any meeting. Except as otherwise required by law or these Bylaws, the affirmative vote of a majority of the Members voting in person or by proxy at a meeting attended by a quorum (present in person or by proxy) shall constitute action on behalf of the Members.

VI. (A) Inasmuch as the time and place of the Annual Meeting of Members are fixed by these Bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of Members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least 15 days before such meeting, to each Member at his or her address as shown on the records of the Corporation.

(B) Any meeting of the Members may be adjourned to any other time and place by the vote of a majority of those Members present or represented at the meeting, whether or not such Members constitute a quorum. It shall not be necessary to notify any Member of any adjournment.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated. Notice of Trustees' meetings may be given orally, by telephone, telegraph or in writing; and notice given in time to enable the Trustees to attend, or in any case notice sent by mail or telegraph to a Trustee's usual or last known place of residence, at least one week before the meeting shall be sufficient. Notice of a meeting need not be given to any Trustee if a written waiver of notice, executed by him before or after the meeting is filed with the records of the meeting, or if he shall attend the meeting without protesting prior thereto or at its commencement the lack of notice to him.

VIII. (A) There shall be four groups of Trustees:

(1) Trustees (the "Corporate Trustees") elected by the Members according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Trustees shall be divided into four classes of six, one class to be elected year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms.

(2) Trustees ("Board Trustees") elected by the Trustees then in office according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Board Trustees shall be divided into four classes of three, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms. It is contemplated that, unless otherwise determined by the Trustees for good reason, Board Trustees shall be individuals who have not been considered for election as Corporate Trustees.

(3) Trustees ex officio, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(4) Trustees emeriti who shall include any Member who has attained the age of seventy years (or the age of sixty five and has retired from his home institution) and who has served a full elected term as a regular Trustee, provided he signifies his wish to serve the Laboratory in that capacity. Any Trustee who qualifies for emeritus status shall continue to serve as a regular Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Members or by the Board, as the case may be. The Trustees ex officio and emeriti shall have all the rights of the Trustees, except that Trustees emeriti shall not have the right to vote.

(B) The aggregate number of Corporate Trustees and Board Trustees elected in any year (excluding Trustees elected to fill vacancies which do not result from expiration of a term) shall not exceed nine. The number of Board Trustees so elected shall not exceed three and unless otherwise determined by vote of the Trustees, the number of Corporate Trustees so elected shall not exceed six.

(C) The Trustees and Officers shall hold their respective offices until their successors are chosen in their stead.

(D) Any Trustee may be removed from office at any time with or without cause, by vote of a majority of the Members entitled to vote in the election of Trustees; or for cause, by vote of two-thirds of the Trustees then in office. A Trustee may be removed for cause only if notice of such action shall have been given to all of the Trustees or Members entitled to vote, as the case may be, prior to the meeting at which such action is to be taken and if the Trustee so to be removed shall have been given reasonable notice and opportunity to be heard before the body proposing to remove him.

(E) Any vacancy in the number of Corporate Trustees, however arising, may be filled by the Trustees then in office unless and until filled by the Members at the next Annual Meeting. Any vacancy in the number of Board Trustees may be filled by the Trustees.

(F) A Corporate Trustee or a Board Trustee who has served an initial term of at least two years duration shall be eligible for re-election to a second term, but shall be ineligible for re-election to any subsequent term until two years have elapsed after he last served as Trustee.

IX. (A) The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall annually elect a Treasurer who shall serve until his successor is selected and qualified. They shall elect a Clerk (a resident of Massachusetts) who shall serve for a term of four years. Eligibility for re-election shall be in accordance with the content of Article VIII(F) as applied to corporate or Board Trustees. They shall elect Board Trustees as described in Article VIII(B). They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents of the Corporation and may remove them at any time. They may fill vacancies occurring in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number as provided in Article X, and to delegate to such Committee such of their own powers as they may deem expedient in addition to those powers conferred by Article X. They shall from time to time elect Members to the Corporation upon such terms and conditions as they shall have determined, not inconsistent with law or these Bylaws.

(B) The Board of Trustees shall also have the power, by vote of a majority of the Trustees then in Office, to elect an Investment Committee and any other committee and, by like vote, to delegate thereto some or all of their powers except those which by law, the Articles of Organization or these Bylaws they are prohibited from delegating. The members of any such committee shall have such tenure and duties as the Trustees shall determine; provided that the Investment Committee, which shall oversee the management of the Corporation's endowment funds and marketable securities, shall include the Chairman of the Board of Trustees, the Treasurer of the Corporation, and the Chairman of the Corporation's Budget Committee, as ex officio members, together with such Trustees as may be required for not less than two-thirds of the Investment Committee to consist of Trustees. Except as otherwise provided by these Bylaws or determined by the Trustees, any such committee may make rules for the conduct of its business; but, unless otherwise provided by the Trustees or in such rules, its business shall be conducted as nearly as possible in the same manner as is provided by these Bylaws for the Trustees.

X. (A) The Executive Committee is hereby designated to consist of not more than ten members, including the ex officio Members (Chairman of the Board of Trustees, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and the affirmative vote of a majority of those voting at any meeting at which a quorum is present shall constitute action on behalf of the Executive Committee. The Executive Committee shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by vote of the Board or by law. The Executive Committee may also appoint such committees, including persons who are not Trustees, as it may from time to time approve to make recommendations with respect to matters to be acted upon by the Executive Committee or the Board of Trustees.

(D) The Executive Committee shall keep appropriate minutes of its meetings and its action shall be reported to the Board of Trustees.

(E) The elected Members of the Executive Committee shall constitute as a standing "Committee for the Nomination of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

XI. A majority of the Trustees, the Executive Committee, or any other committee elected by the Trustees shall constitute a quorum; and a lesser number than a quorum may adjourn any meeting from time to time without further notice. At any meeting of the Trustees, the Executive Committee, or any other committee elected by the Trustees, the vote of a majority of those present, or such different vote as may be specified by law, the Articles of Organization or these Bylaws, shall be sufficient to take any action.

XII. Any action required or permitted to be taken at any meeting of the Trustees, the Executive Committee or any other committee elected by the Trustees as referred to under Article IX may be taken without a meeting if all of the Trustees or members of such committee, as the case may be, consent to the action in writing and such written consents are filed with the records of meetings. The Trustees or members of the Executive Committee or any other committee appointed by the Trustees may also participate in meeting by means of conference telephone, or otherwise take action in such a manner as may from time to time be permitted by law.

XIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such a manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees then in office.

XIV. These Bylaws may be amended by the affirmative vote of the Members at any meeting, provided that notice of the substance of the proposed amendment is stated in the notice of such meeting. As authorized by the Articles of Organization, the Trustees, by a majority of their number then in office, may also make, amend, or repeal these Bylaws, in whole or in part, except with respect to (a) the provisions of these Bylaws governing (i) the removal of Trustees and (ii) the amendment of these Bylaws and (b) any provisions of these Bylaws which by law, the Articles of Organization or these Bylaws, requires action by the Members.

No later than the time of giving notice of the meeting of Members next following the making, amending or repealing by the Trustees of any Bylaw, notice thereof stating the substance of such change shall be given to all Corporation Members entitled to vote on amending the Bylaws.

Any Bylaw adopted by the Trustees may be amended or repealed by the Members entitled to vote on amending the Bylaws.

XV. The account of the Treasurer shall be audited annually by a certified public accountant.

XVI. Except as otherwise provided below, the Corporation shall, to the extent legally permissible, indemnify each person who is, or shall have been, a Trustee, director or officer of the Corporation or who is serving, or shall have served, at the request of the Corporation as a Trustee, director or officer of another organization in which the Corporation directly or indirectly has any interest, as a shareholder, creditor or otherwise, against all liabilities and expenses (including judgments, fines, penalties and reasonable attorneys' fees and all amounts paid, other than to the Corporation or such other organization, in compromise or settlement) imposed upon or incurred by any such person in connection with, or arising out of, the defense or disposition of any action, suit or other proceeding, whether civil or criminal, in which he or she may be a defendant or with which he or she may be threatened or otherwise involved, directly or indirectly, by reason of his or her being or having been such a Trustee, director or officer.

The Corporation shall provide no indemnification with respect to any matter as to which any such Trustee, director or officer shall be finally adjudicated in such action, suit or proceeding not to have acted in good faith in the reasonable belief that his or her action was in the best interests of the Corporation. The Corporation shall provide no indemnification with respect to any matter settled or compromised, pursuant to a consent decree or otherwise, unless such settlement or compromise shall have been approved as in the best interests of the Corporation, after notice that indemnification is involved, by (i) a disinterested majority of the Board of Trustees or of the Executive Committee or, (ii) a majority of the Corporation's Members.

Indemnification may include payment by the Corporation of expenses in defending a civil or criminal action or proceeding in advance of the final disposition of such action or proceeding upon receipt of an undertaking by the person indemnified to repay such payment if it is ultimately determined that such person is not entitled to indemnification under the provisions of this Article XVI, or under any applicable law.

As used in this Article, the terms "Trustee," "director" and "officer" include their respective heirs, executors, administrators and legal representatives, and an "interested" Trustee, director or officer is one against whom in such capacity the proceeding in question or another proceeding on the same or similar grounds is then pending.

To assure indemnification under this Article of all persons who are determined by the Corporation or otherwise to be or to have been "fiduciaries" of any employee benefit plan of the Corporation which may exist from time to time, this Article shall be interpreted as follows: (i) "another organization" shall be deemed to include such an employee benefit plan, including, without limitation, any plan of the Corporation which is governed by the Act of Congress entitled "Employee Retirement Income Security Act of 1974," as amended from time to time ("ERISA"); (ii) "Trustee" shall be deemed to include any person requested by the Corporation to serve as such for an employee benefit plan where the performance by such person of his or her duties to the Corporation also imposes duties on, or otherwise involves services by, such person to the plan or participants or beneficiaries of the plan; (iii) "fines" shall be deemed to include any excise taxes assessed on a person with respect to an employee benefit plan pursuant to ERISA; and (iv) actions taken or omitted by a person with respect to an employee benefit plan in the performance of such person's duties for a purpose reasonably believed by such person to be in the interest of the participants and beneficiaries of the plan shall be deemed to be for a purpose which is in the best interests of the Corporation.

The right of indemnification provided in this Article shall not be exclusive of or affect any other rights to which any Trustee, director or officer may be entitled under any agreement, statute, vote of members or otherwise. The Corporation's obligation to provide indemnification under this Article shall be offset to the extent of any other source of indemnification or any otherwise applicable insurance coverage under a policy maintained by the Corporation or any other person. Nothing contained in this Article shall affect any rights to which employees and corporate personnel other than Trustees, directors or officers may be entitled by contract, by vote of the Board of Trustees or of the Executive Committee or otherwise.

VI. REPORT OF THE DIRECTOR

I have the pleasure of writing a Director's Report which is concerned in large part with plans for the future, rather than with analysis of the past. My pleasure implies neither a lack of interest in the past nor any uneasy omen. It comes, rather, from my review of those tall stacks of paper recording the events, the finances, the problems, and the accomplishments—scientific and administrative—of the past year, a review that always precedes the writing of these reports. There it becomes evident that on most fronts of MBL operations there are meetings, studies, and planning exercises directed toward the post-Centennial decade. How fundamental a change this represents in the state of the place can be appreciated only by those who know something of the MBL's past.

Such activity means that we see no threats or difficulties, in things as they are, grave enough to inhibit creative anticipation. It means that MBL people are sufficiently confident of the health of the enterprise to give time to imagining an exciting future, rather than to considering how to survive. It was not always thus. I am able therefore to write of plans and goals, confident that the remaining content of this volume will be at least reassuring, and possibly even a source of pride, to members of the Corporation.

Committees

1985 was MBL Centennial Year minus three, none too soon to begin to plan for the events of that celebration. The Trustees having reviewed and approved a proposed structure for a committee to plan and execute the events, the structure was built, with people, in the course of the year. Overseeing all Centennial-related activities will be a Centennial Committee, which includes among its distinguished members James D. Ebert as Chairman. We are doubly fortunate in Ebert's acceptance of the responsibility: he is not only a devoted Corporation member and the Laboratory's President-Director *emeritus*, but also a scientist and administrator of international stature.

The oversight committee includes, *inter alia*, the Chairmen of four subcommittees, each of which has been empaneled and is hard at work. In charge of the Subcommittee on History is Garland Allen. Raymond E. Stephens chairs the Subcommittee on Events, and "events" is meant to cover not only the necessary celebratory occasions of 1988, but scientific meetings of international significance, beginning in 1987. An expert and enthusiastic Subcommittee on Public Information is headed by author and friend of the Laboratory John Pfeiffer. And finally, financial management of the Centennial celebrations, *in extenso,* is the responsibility of a Finance Subcommittee headed by D. Thomas Trigg.

I like to believe that it is a measure of the MBL's future, as well as of its remarkable history, that we are able to assemble so talented a group of volunteers to work for an institution that is not, after all, among those listed as best-endowed, nor is it constantly in the public eye.

By the reorganization of old committees and the creation of several new ones, the Trustees have signaled a new determination to manage the MBL seriously and well—as though, in fact, it were itself well and to be taken seriously, in the way that large and complex intellectual enterprises must today be taken if they are to succeed. Thus we have in the course of the year sharpened the charge of the Investment Committee, expanded and systematized the work of the Compensation Committee, created a new and professionally qualified Audit Committee, and empaneled a Committee on Laboratory Goals.

Investment, the first of these, has been in existence for a good many years and it has given the MBL invaluable service. But with our investments now in excess of $11 million, it is important for all levels of decision-making to be identified and monitored, and for the many choices that have to be made each year to be made on a reliable schedule, recorded promptly, and implemented by timely actions of the MBL administration and the investment management firm. All these needs are dealt with under the revised committee charge. The members have accepted it.

As the MBL's still-skeletal administrative capability is enhanced, it becomes necessary for the compensation of managers—a category that includes all the Department heads—to be reviewed with as much care, knowledge of market forces, and attention to individuals as has always been the case for non-administrative employees. The new Compensation Committee will see to that.

As recently as 1978, when the business and academic worlds were well into the second generation of administrative data processing by electronic means, the MBL was still doing all its business with pencil and paper. It did not take much effort to convince the community that the time had come for conversion to electronic data processing. The conversion was made coincident with reorganization of the accounting functions under the Controller's office. Subsequent improvements in financial management have been evident to nearly everyone associated with the Laboratory. We are, however, at the junction of two very fast-flowing streams: the technology itself, and the increasingly complex financial operations of the MBL. In the future we will require not only computer-based accounting, but *excellent* accounting at that, and incisive data analysis for management and planning. The MBL must also have regular and more expert liaison with its external auditors, those of government and foundation offices as well as those we employ for the annual review of our books. The new Audit Committee is superbly qualified for these purposes. It will work with the Controller, henceforth, as well as with the Director and all the Trustees, to whom it will ultimately report.

The last comprehensive plan for the Laboratory was written in 1978 and adopted by the Trustees in the winter of 1979. It was a comprehensive plan in the sense that it set goals in each of the MBL missions: summer research, year-round research, instruction; and it did so in the context of existing resources and reasonable projections of their growth. It was a plan meant to serve, with modification and amendment as needed, up to the Centennial, give or take a year. As it happens, this was the first, as well as the last, such long-term plan for the institution.

The 1979 plan, and all the work of review, data analysis, and feasibility testing that went into it, were done by the then-new Director. It could not have been otherwise:

differences of opinion within the Corporation, even on fundamental issues, were too great, and organizational and administrative resources too small, to have allowed the Corporation, speaking via its Trustees, to do the job.

We have succeeded with what is, by any reasonable standard, a remarkable fraction of the 1979 plan. It is a fair expectation that the essentials, save perhaps a Marine Resources Center, will have been realized by 1988. To be sure, some of the elements have had to be modified. Seven years is a very long time in modern biology, and in the history of relations between government and independent research laboratories. But most of the modifications have been upward in scale, not downward, and most of those, too, have been achieved or will be achieved in the near future.

It is time for a new MBL plan. Even if we were in a field less dynamic and more predictable than biomedical science, we should need one: seven years is an ancient guideline for sticking to an undertaking. Thereafter, in response to the inevitable itch, there must be some scratching. Sensible people and institutions anticipate the itch and regulate the scratch.

The MBL is now strong enough. There is sufficient loyalty and unanimity on what were recently divisive issues to allow the next plan to be made by those who *should* be its architects: the Corporation and the Trustees themselves. It is not that the Director is alien to either body: he is one of both, and by the nature of things will always, whoever he or she may be, have unlimited access to their wisdom and their beliefs (not all of which are necessarily wise). But in an institution as horizontal as the MBL, and in so explosively growing a business as biomedical science, no comprehensive plan should be authored by one person.

Machinery for making the next comprehensive plan was established during the 1985–86 year. It begins with a new Trustees' Committee, the Committee on Laboratory Goals, chaired by Gerald Fischbach. In its initial form the Committee consists entirely of Corporation members: as its inquiries are broadened during the summer of 1986, members and consultants will be added according to need. This Committee has a simple but very weighty charge: to recommend to the Trustees directions and standards for all MBL scientific programs in the decade following the Centennial. The recommendations are to be based upon exhaustive analysis of current resources, probable future resources, special strengths and opportunities in the existing configuration, actual or potential weaknesses, and, insofar as is possible, an adherence to the founding principles of the Laboratory. It is perhaps well to list the relevant ones here:

Independence;
Avoidance of identification with any single biological discipline;
Emphasis upon intellectual quality, as opposed to mere utility;
Maximum utilization of resources of the sea for research, but no exclusive commitment to marine biology for its own sake;
Instruction and scientific communication as important as private, individual research.

Dr. Fischbach's carefully assembled and highly qualified committee is at work: every Corporation member will be hearing from and of it before long. In the end, the product of its arguments and eventual agreements will change the shape of the MBL as much as, perhaps more than, did the Director's plan of 1979. This is as it should be: a biological institution whose spectrum of interests does not change within a decade—in these waning decades of the twentieth century—cannot be a part of the action of life science.

All the rest

Nothing else *needs* to be said in this year's Director's Report. The facts are in the book. Value judgments are implicit in the continued fluorishing of MBL programs, even though we are in times and circumstances such that other institutions, better-endowed and with missions more easily featured in Sunday Supplements, have fallen into deep trouble. I can afford to report in pastel—a set of strokes meant to suggest reality, rather than to render it.

Year-round research: Shinya Inoué's superb book, product of his years as a resident MBL scientist fully engaged in research, is published. It is the definitive text of the new technology of video microscopy, and that, in turn is an MBL story. Tom Reese will give a Friday Evening Lecture in 1986: its title will be *Kinesin—an MBL Project.* The Ecosystems Center, now led by John Hobbie, installs a sophisticated mass spectrometry facility for its own and general use, reflecting increased exploitation of stable isotope methods in ecological research. The facility is funded by generous grants from the NSF and the A. W. Mellon Foundation, and to some extent by the Center's own reserve funds. The productivity of Louis Leibovitz's Laboratory of Marine Animal Health continues to rise: a science of marine animal medicine—to include invertebrates as well as vertebrates—is in the making. The Laboratories of Sensory Physiology and of Biophysics continue to be identified by various forms of recognition as excellent. Serious planning for the future of the Boston University Marine Program appears to have begun: searches for a new Director and additional faculty are underway. The program continues to attract excellent graduate students. In these times of insanely chancy funding, such distinguished MBL investigators as J. R. Whittaker, R. E. Stephens, and O. Shimomura continue to be solidly supported. L. Jaffe's National Vibrating Probe facility provides more and more, not only for Jaffe's important research, but for others who discover how useful is the unique field-mapping capability of the instrument. The whole year-round scientific community seems, despite the peculiar arrangements by which it exists at the MBL, to be productive, mutually cooperative, even, possibly, happy.

Summer research and instruction: The merest vignette, but one that should suffice for Corporation members, most of whom have a sense of how things are in this era of federal deficit reduction: in 1986 the MBL will again be full. At least at the time of writing (May, 1986), there is no suggestion of a decline of demand for research space, instruction, conference facilities. Applications for places in summer courses are up by more than 30% over 1985. The Friday Evening Lectures will be as distinguished as ever; there will be more fellows and scholars; more short-term visitors to courses and to the scientific community as a whole. Not unimportant: there will be twenty new, very handsome rental cottages: every one will be filled: there is a waiting list already.

The departments: Here is the most attenuated vignette of all. Hurricane Gloria. In September of 1985, with barely adequate warning, the storm was upon us, and at a time of year when there is an understandable relaxation—a slight let-down of the guard—among MBL employees, catching a communal breath after the frenetic summer. To be sure, Gloria could have been much nastier with the northeast USA than she was. But there was nevertheless a great deal of damage everywhere along the coast, and Woods Hole was within the target area.

On an already wet and evil morning, not long after dawn: I find the MBL a beehive. B & G staff, Aparatus staff, John Valois and the Captains, research assistants—everyone with responsibility (including, of course, a cadre of anxious scientists, torn between fears for the supercold freezer in the laboratory and worries about the food freezer—and the children—at home). A beehive literally. What might be taken for a lot of senseless milling about is in fact the worker bees in a *schwäntzetanz:* moving purposefully, communicating, putting forward the imperative of the hive. All vessels afloat are secured; heavy storm shutters are fixed in place; scores of sandbags are brought and emplaced; emergency power provided for; glass area protected. Not long after, the first great blasts strike . . . 50 knots . . . and there are creakings and the groans of tied-down structure under strain. Water Street begins to live up to its name as waves break green and white over the sea wall. We are lucky: the tide is falling and will continue to fall through the peak of wind velocity, which ought to be at about noon. At noon in fact it seems the late evening of a vicious winter's day. My hand anemometer, borrowed from the nav-station of UCA, my boat (a pugnacious fiddler-crab is UCA), registers 75 knots. The long day ends. It is still blowing crazily, trees bending as though they were mere sticks; but it is clear that the MBL will be all right. The next day is partly sunny and warm. The sea is innocent and seems to know nothing about violence and danger.

Practically no words were said, before or after, by anyone behaving as a drill-sergeant. MBL people simply did what they know how to do, when it had to be done. The damage was, in the end and when assessed, minor. As is always the case here, we learned a little about how to do things better the next time.

VII. REPORT OF THE TREASURER

In February of 1986, I discussed with Dr. Gifford my wish to be replaced as Treasurer of the Marine Biological Laboratory. This decision on my part was reached after thoughtful inquiry, one year "on the job," and my judgment that the responsibilities of the office of the Treasurer, stated and implied, require full-time "hands-on" attention and management.

Today the Laboratory operates year-round. It has financial transactions with entities both private and public. Its financial health depends on its continuing ability to attract grants and gifts for the purchase and maintenance of its facilities and equipment.

To meet the current demand, the Laboratory engages in many "businesses." The financial consequences of these businesses and the time it takes to manage them varies widely. Many of these responsibilities should not be delegated nor should they be managed on a "knee jerk" reaction basis by a part-time volunteer Treasurer. I have decided to step aside in support of this judgment.

My review of prior years' Treasurer's reports to the Corporation shall be my guide in the preparation of this report.

First, I shall comment on the *Statement of Assets and Liabilities* (including fund balances). Then I shall comment briefly on the *Schedule of Support, Revenues, Expenses, and Changes in the Fund Balance.*

Net current assets (Working Capital) decreased $482,000 occasioned by a decrease in cash and cash equivalents of $600,000, of which $450,000 was transferred to the Pooled Investment portfolio; the increase in Receivables of $441,000 mostly in grants, includes $211,000 of billings for which payment was received subsequent to the year-end closing of the books. Efforts to reduce the grants and other receivables have been underway and I am pleased to report that these receivables have been reduced from $1,115,000 to $497,000 as of the end of March, 1986.

Accounts payable increased $174,000. Note Payable of $100,000 represents the proceeds of a temporary unsecured loan. These proceeds were used for the Memorial Circle construction pending the recording of and repayment from the permanent secured loan. This occurred in January of 1986. Two new items were added to the schedule in 1985; a contingent liability of $50,000 for the over-recovery of overhead costs and an accrual of $75,000 for wages earned during the latter part of the year, which were paid in 1986.

Investments (including retirement fund securities) increased $861,000 (cost value) principally reflecting the first increment of $500,000 from the Mellon Match commitment.

MBL books its investments at cost. (Not all non-profit corporations do so.) Given the remarkable advance in stock and bond values in the last quarter of 1985, the year-end book value of the endowment (including quasi-endowments) and the retirement funds is understated by $2,254,000.

The Statement of Support, Revenues, Expenses, and Changes in Fund Balance shows a significant increase in expenses of $1,000,000—an increase of 10%, while income to support these expenses increased $300,000 or 2.8%. I am advised that the 1985 Budget prepared and approved in 1984 anticipated the increased expense.

In summary, if one includes the positive influence of the securities market on the financial position of the MBL as of year-end 1985, it was on average a very good year for the MBL.

The financial control unit under the direction of John Speer and with the assistance of Coopers & Lybrand, our independent auditors, and other counsel, have been engaged for some time in developing improvements in the accounting, personnel management, and cost control systems of the MBL. During 1985, three significant recommendations were presented to the Executive Committee and received their approval. They were:

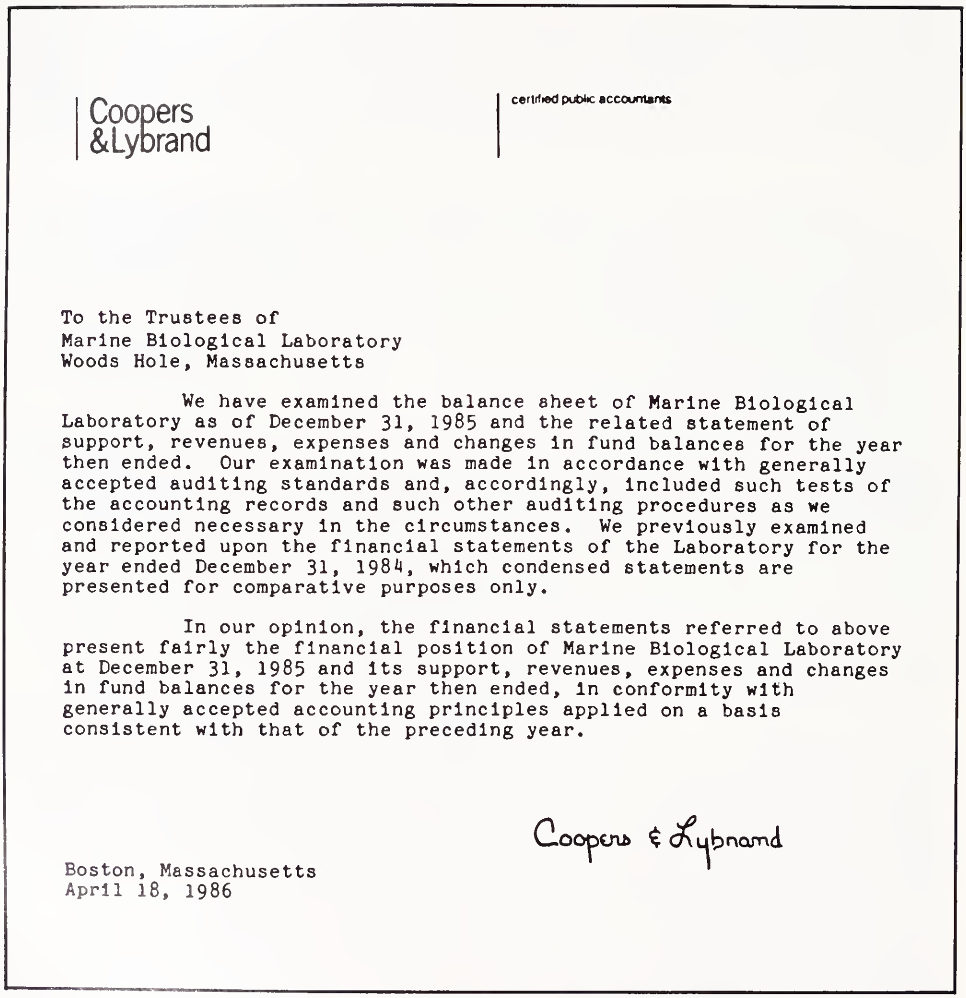
(a) An improved overhead assignment formula that will ensure the recovery of a fair division of these costs from the various users of the facilities and equipment.
(b) A personnel management system which includes individual job descriptions and evaluations. These will be most useful in providing standards for logical and equitable management and compensation to our staff.
(c) The establishment of separate funds in the financial accounting system for housing and for food services. These new restricted accounts will appear in the 1986 financial reports.

Under "Other but Necessary Business": the twenty units of additional housing at Memorial Circle were authorized and commenced in the fall of 1985 with completion scheduled for June, 1986. I am pleased to report that they have been finished on time and within budget. The placement of these new units has been the subject of favorable trustee comment. Credit for this excellent work belongs to our engineers, Holmes & McGrath, who developed the site plan and utility design; Woodside Park Corporation for construction of the buildings, roads, etc.; to Homer Smith for his continuing assistance at all levels including the procurement of the furnishings for the cottages; and to Donald Lehy, the owner's representative at the site.

In 1985, Dr. Gross sought and obtained multiple year gifts for the support of the instruction program. These gifts are sufficient to balance instruction costs through 1987. This was a most necessary life sustaining transfusion for this function. From where and from whom will funds be obtained to replace those funds when they are expended as planned? When will our endowments increase in principal value so they will generate sufficient income to replace our dependency on crisis funding for ongoing functions? These are questions that should and must be answered by the senior management, Trustees, Corporators, and the Associates of MBL.

This brings me full circle to my opening remarks. Times have changed. Financial planning and its execution is a full-time, year-round responsibility of the MBL. The corporate office charged with this responsibility is and has been that of the Treasurer, when in fact, the duties have been performed on a daily basis by a non-corporate officer, the Controller. It is my conviction that this arrangement should be changed and that the MBL must have a full-time senior financial officer.

While I have been preparing these comments, I have learned that Robert D. Manz recently of Coopers & Lybrand and for the past several years the auditor in charge of the MBL audit, has recently established himself as a financial consultant in private practice. Bob has agreed to become MBL's Treasurer and will bring to the office a board knowledge of the financial affairs of non-profit, scientific research organizations and in the specific, a unique knowledge of the strengths and weaknesses of MBL's financial affairs.



Coopers
&Lybrand

certified public accountants

To the Trustees of
Marine Biological Laboratory
Woods Hole, Massachusetts

We have examined the balance sheet of Marine Biological Laboratory as of December 31, 1985 and the related statement of support, revenues, expenses and changes in fund balances for the year then ended. Our examination was made in accordance with generally accepted auditing standards and, accordingly, included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We previously examined and reported upon the financial statements of the Laboratory for the year ended December 31, 1984, which condensed statements are presented for comparative purposes only.

In our opinion, the financial statements referred to above present fairly the financial position of Marine Biological Laboratory at December 31, 1985 and its support, revenues, expenses and changes in fund balances for the year then ended, in conformity with generally accepted accounting principles applied on a basis consistent with that of the preceding year.

Coopers & Lybrand

Boston, Massachusetts
April 18, 1986

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1985 and 1984

Assets	*1985*	*1984*
Cash and savings deposits	$ 197,668	$ 214,449
Money market securities (Note F)	225,000	825,000
Accounts receivable, net of allowance for uncollectible accounts	374,301	278,529
Receivables due for costs incurred on grants and contracts	741,206	395,788
Other assets	11,859	14,855
Total current assets	1,550,034	1,728,621
Investments, at cost (Note F)	8,881,213	8,020,168
Land, buildings and equipment (Note C)	17,805,051	17,436,604
Less accumulated depreciation	(6,579,654)	(6,090,140)
	11,225,397	11,346,464
Total assets	$21,656,644	$21,095,253

Liabilities and Fund Balances	*1985*	*1984*
Accounts payable and accrued expenses	$ 448,281	$ 273,973
Deferred income	124,932	95,519
Note payable (Note H)	100,000	—
Total current liabilities	673,213	369,492
Construction accounts payable	58,496	
Current unrestricted funds	44,586	13,952
Current restricted funds:		
Unexpended grants	180,905	410,210
Unexpended gifts	424,727	305,837
Unexpended income of endowment funds	31,619	18,736
	637,251	734,783
Endowment funds:		
Unrestricted purposes	1,805,942	1,771,188
Restricted purposes	1,621,423	1,432,604
	3,427,365	3,203,792
Quasi-endowment funds:		
Unrestricted purposes	949,600	1,020,338
Restricted purposes	2,889,970	2,692,957
	3,839,570	3,713,295
Plant funds:		
Unrestricted	11,084,571	11,346,464
Restricted	56,669	105,748
	11,141,240	11,452,212
Retirement fund balance (Note D)	1,834,923	1,607,727
Total liabilities and fund balances	$21,656,644	$21,095,253

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENT OF SUPPORT, REVENUES, EXPENSES AND CHANGES IN FUND BALANCES

for the year ended December 31, 1985
(with comparative totals for 1984)

	Current Fund		*Endowment Funds*		*Quasi-endowment Funds*		*Plant Funds*				
	Unrestricted	*Restricted*	*Unrestricted*	*Restricted*	*Unrestricted*	*Restricted*	*Unrestricted*	*Restricted*	*Retirement Funds*	*1985 Total All Funds*	*1984 Total All Funds*
Support and revenues:											
Grant reimbursement of direct costs:											
Instruction		$ 171,084								$ 171,084	$ 101,377
Research		3,508,303								3,508,303	3,685,865
Recovery of indirect costs related to research and instruction											
Summer program	$ 468,262									468,262	444,134
Year-round program	1,722,521									1,722,521	1,572,260
Other	66,226									66,226	77,628
Instruction	208,820									208,820	35,131
Tuition		270,237								270,237	247,685
Support activities:											
Dormitory	581,515									581,515	497,263
Dining hall	396,952									396,952	242,404
Library	215,348									215,348	227,551
Biological bulletin	156,245									156,245	112,134
Research services	415,830									415,830	400,284
Marine resources	173,860									173,860	165,000
Other	(3,483)									(3,483)	472
Investment income	287,821	210,123							$ 112,675	610,619	618,665
Realized gains (losses) on investments		(344)	$ 34,754	$ (5,426)	(3,597)	45,431			27,266	98,084	483,380
Addition to fund							$ 29,381		146,253	175,634	105,328
Gifts	106,978	1,526,550		29,290						1,662,818	1,613,240
Miscellaneous revenue	112,716									112,716	78,082
Total support and revenues	4,909,611	5,685,953	34,754	23,864	(3,597)	45,431	29,381		286,194	11,011,591	10,707,883

Expenses:											
Instruction		1,063,601								1,063,601	787,063
Research	45,388	4,002,757								4,048,145	4,197,569
Scholarships and stipends		299,793								299,793	190,280
Payments to pensioners									56,104	56,104	39,285
Support activities:											
Dormitory	382,655									382,655	254,695
Dining hall	388,712									388,712	245,141
Library	480,881									480,881	452,500
Biological bulletin	153,593									153,593	158,127
Research services	720,509									720,509	635,897
Marine resources	375,450									375,450	325,660
Administration	986,811									986,811	816,719
Sponsored projects administration	165,269	101,515								266,784	256,257
Plant operations	1,023,293									1,023,293	987,992
Depreciation							489,517			489,517	456,394
Other	—	72,750							3,819	76,569	14,816
Total expenses	4,722,561	5,540,416	—	—	—	—	489,517	—	59,923	10,812,417	9,818,395
Excess (deficit) of support and revenues over expenses	187,050	145,537	34,754	23,864	(3,597)	45,431	(460,136)	—	226,271	199,174	889,488
Transfers among funds:											
Acquisition of fixed assets	(87,468)	(61,696)					198,243	(49,079)		—	—
Mellon match transfers	(54,878)	(110,077)		164,955						—	—
Ecosystem investment income reinvested		(151,582)				151,582					
Other transfers	(14,070)	80,286	—	—	(67,141)	—	—	—	925	—	—
Total transfers among funds	(156,416)	(243,069)	—	164,955	(67,141)	151,582	198,243	(49,079)	925	—	—
Net change in fund balances	30,634	(97,532)	34,754	188,819	(70,738)	197,013	(261,893)	(49,079)	227,196	199,174	889,488
Fund balances, beginning of year	13,952	734,783	1,771,188	1,432,604	1,020,338	2,692,957	11,346,464	105,748	1,607,727	20,725,761	19,836,273
Fund balances, end of year	$ 44,586	$ 637,251	$1,805,942	$1,621,423	$ 949,600	$2,889,970	$11,084,571	$ 56,669	$1,834,923	$20,924,935	$20,725,761

The accompanying notes are an integral part of the financial statements.

Notes to Financial Statements

A. *Purpose of the Laboratory:*

The purpose of Marine Biological Laboratory (the "Laboratory") is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and nature history.

B. *Significant Accounting Policies:*

Basis of Presentation—Fund Accounting

In order to ensure observance of limitations and restrictions placed on the use of resources available to the Laboratory, the accounts of the Laboratory are maintained in accordance with the principles of "fund accounting." This is the procedure by which resources are classified into separate funds in acccordance with specified activities or objectives. In the accompanying financial statements, funds that have similar characteristics have been combined.

Externally restricted funds may only be utilized in accordance with the purposes established by the donor or grantor of such funds. However, the Laboratory retains full control over the utilization of unrestricted funds. Restricted gifts, grants, and other restricted resources are accounted for in the appropriate restricted funds. Restricted current funds are reported as revenue when expended for current operating or other purposes. Unrestricted current funds are reported as revenue when earned.

Endowment funds are subject to restrictions requiring that the principal be invested with income available for use for restricted or unrestricted purposes by the Laboratory. Quasi-endowment funds have been established by the Laboratory for the same purposes as endowment funds; however, the principal of these funds may be expended for various restricted and unrestricted purposes.

Reclassifications

The financial statements for 1985 reflect certain changes in classification of revenue, expenses, and changes in fund balances. Similar reclassifications have been made to amounts previously reported in order to provide consistency of the financial statements.

Contracts and Grants:

Revenues associated with contracts and grants are recognized in the Statement of support, revenues, and expenses, and changes in fund balances when received and as related costs are incurred. The Laboratory reimbursement of indirect costs relating to government contracts and grants is based on negotiated indirect cost rates with adjustments for actual indirect costs in future years. Any over- or under-recovery of indirect costs is recognized through future adjustments of indirect cost rates.

Investments

Investments purchased by the Laboratory are carried at cost. Money market securities are carried at cost which approximates market. Investments donated to the Laboratory are carried at fair market value at the date of the gift. For determination of gain or loss upon disposal of investments, cost is determined based on the average cost method.

Investment Income and Distribution

The Laboratory follows the accrual basis of accounting except that investment income is recorded on a cash basis. The difference between such basis and the accrual basis does not have a material effect on the determination of investment income earned on a year-to-year basis.

Investment income includes income from the investments of specific funds and from the pooled investment account. Income from the pooled investment account is distributed to the participating funds on the basis of their proportionate share at market value adjusted for any additions or disposals to pooled funds.

C. *Land, Buildings, and Equipment:*

The following is a summary of the unrestricted plant fund assets:

	1985	*1984*
Land	$ 689,660	$ 689,660
Construction in progress	140,826	—
Buildings	14,861,244	14,772,449
Equipment	2,113,321	1,974,495
	17,805,051	17,436,604
Less accumulated depreciation	6,579,654	6,090,140
	$11,225,397	$11,346,464

Depreciation is computed using the straight-line method over estimated useful lives of fixed assets.

D. *Retirement Fund:*

The Laboratory has a noncontributory pension plan for substantially all full-time employees, which complies with the requirements of the Employee Retirement Income Security Act of 1974. The Laboratory's policy is to fund pension costs accrued, as determined under the projected unit credit method. The actuarially determined pension expense charged to operations in 1985 was $146,253. As of the latest valuation date, based on benefit information at December 31, 1984, the actuarial present values of vested and nonvested benefits, assuming an investment rate of return of 7%, were approximately $1,300,175 and $60,833, respectively. At December 31, 1985, net assets of the plan available for benefits were approximately $1,687,072.

In addition, the Laboratory participates in the pension program of the Teachers Insurance and Annuity Association.

E. *Pledges and Grants:*

As of December 31, 1985 and 1984, the following amounts remain to be received on gifts and grants for specific research and instruction programs, and are expected to be received as follows:

	December 31, 1985		*December 31, 1984*	
	Unrestricted	*Restricted*	*Unrestricted*	*Restricted*
1986	$15,000	$ 703,128	$5,000	$ 8,000
1987	10,000	550,240	—	6,651
1988	10,000	15,000		
	$35,000	$1,268,368	$5,000	$14,651

In February 1979, the Laboratory initiated the MBL Second Century Fund, a phased effort, to secure $23,000,000 in support of capital rehabilitation, new construction, and endowment funds. As of December 31, 1985, the Laboratory has received pledges related to this effort of approximately $8,550,129, of which a substantial portion has been collected.

F. *Investments:*

The following is a summary of the cost and market value of investments at December 31, 1985 and 1984 and the related investment income and disposition of investment income for the years ended December 31, 1985 and 1984.

	Cost		*Market*		*Investment Income*	
	1985	*1984*	*1985*	*1984*	*1985*	*1984*
Endowment, Quasi-endowment, and Retirement Funds						
U. S. Government securities	$2,370,717	$2,422,701	$ 2,495,164	$2,405,964	$253,156	$272,442
Corporate fixed income obligations	999,333	749,926	1,015,671	707,678	74,169	47,570
Common stocks	4,620,228	4,073,866	6,720,525	4,977,757	220,892	203,158
Preferred stock	2,860	12,549	9,183	22,940	1,799	1,798
Money market securities	872,326	745,377	879,218	745,377	48,853	82,636
Real estate	15,749	15,749	15,749	15,749	—	—
Total	8,881,213	8,020,168	11,135,510	8,875,465	598,869	607,604
Less custodian fees					(27,741)	(32,331)
					571,128	575,273
Unrestricted Current Fund						
Money market securities	225,000	825,000	225,000	825,000	39,491	43,392
Total investments	$9,106,213	$8,845,168	$11,360,510	$9,700,465	$610,619	$618,665
Disposition of investment income:						
Restricted for current use:						
Utilized in current operations					193,131	261,257
Available for future operations					16,992	(9,065)
Total restricted current and quasi-endowment funds					210,123	252,192
Retirement fund					112,675	88,223
Unrestricted—utilized in current operations					287,821	278,250
					$610,619	$618,665

At December 31, 1985 and 1984 the following summarizes the participation of the various funds in the investment pool:

	1985	*1984*
Unexpended income of endowment	$ 31,619	$ 18,736
Unrestricted endowment	1,805,942	1,771,188
Restricted endowment	1,795,745	1,287,561
Unrestricted quasi-endowment	996,078	1,077,988
Restricted quasi-endowment	2,562,520	2,364,407
Retirement	1,689,309	1,500,288
	$8,881,213	$8,020,168

G. *Interfund Borrowings:*

Interfund balances at December 31 are as follows:

	1985	*1984*
Current Funds		
Due to retirement fund	$(146,253)	$(105,328)
Due to plant funds	(56,669)	(105,748)
Due to endowment funds	(194,210)	(500,000)
Due from (to) quasi-endowment funds	120,563	103,365
	$(276,569)	$(607,711)

H. *Indebtedness:*

On December 11, 1985, the Laboratory obtained an unsecured loan in the amount of $100,000 with interest at 10.25% due December 19, 1985, for construction of cottages. This was replaced after December 31, 1985 by a 26 year construction/permanent mortgage loan in the amount of $1.5 million bearing interest based on the bank's prime rate plus three quarters percent (.75%) on a floating basis for the initial five year period with a floor of 7.50% and a ceiling of 13.00%. At the end of the construction phase, principal and interest payments of $15,000 shall be due and payable monthly. The mortgage loan is collateralized by a first mortgage on the land and properties known as Memorial Circle, with recourse in the event of default limited to this land and property and the related revenue.

VIII. REPORT OF THE LIBRARIAN

The National Marine Fisheries Service in Woods Hole has placed its library collection in the MBL Library. We now provide full library services to the scientists at the Fisheries making the MBL Library the main library for all four institutions in Woods Hole. This move has been discussed for years and it became official when the MBL was awarded the contract by NOAA early this year. Our collection will be increased by approximately 1500 books. Most of the journal sets held at the Fisheries are duplicated here and their volumes will be sent to other Fishery libraries. A few new titles will be added to the periodical collection.

The scientists at the Fisheries have used the MBL Library since the founding of the Laboratory. The Minutes of the MBL Trustee meeting in 1889 record that the Fisheries contributed both books and pamphlets to the original collection compiled in 1888. Over the years their scientists have been helpful with advice on collection development in their specialized subjects. Everyone benefits from such an arrangement. The Fisheries will save space and money while at the same time contributing financially to the MBL Library operation.

The Reprint collection (Stack One) was cleared this year for the final move of the periodical collection. We say "final move" as we are committed to a collection of no larger than 200,000 volumes. The new laser disc technology makes it possible to keep adding new material to the collection without requiring more shelf space. The Library Users Committee and the librarians are studying all new developments of this method of electron storage. During August, when a large number of scientists were in residence, we had a Library Week. A number of companies exhibited their products for three days and an evening meeting was held for discussion with all involved.

For three months we moved the periodical collection, using the stack floor gained by the reprint move. All journals are now housed in alphabetical order on the five floors of stacks. The Reprints have been placed in storage until time permits for rearrangement in another area of the library. Ninety percent of the reprints are available in the published volumes in the stacks and all are cataloged in the main catalog by author so the major part of the collection is still available.

This year we started placing our serial collection of nearly 5000 titles into the OCLC database. It's an expensive project but we are fortunate to have all expenses covered by a Title III Grant from the Massachusetts Library Commissioners. Catherine Norton, Assistant Librarian, wrote the grant working with the Librarian at the Falmouth Public Library and 42 other Public libraries on the Cape and Islands. All serial titles held in southeastern Massachusetts will be placed in this database. The information will be available on-line to all libraries. Catherine is the coordinator of the project and a new IBM-PC is in our Library for the specific use of the people involved in entering the material. The computer will remain the property of the Library when the project is completed.

IX. EDUCATIONAL PROGRAMS

SUMMER

BIOLOGY OF PARASITISM

Course directors
Englund, Paul, Johns Hopkins School of Medicine
Sher, Alan, NIAID/NIH

Other faculty, staff, and lecturers

ALLISON, ANTHONY C., Syntex
ANDERS, ROBIN, Walter and Eliza Hall Institute, Australia
BANGS, JAMES D., Johns Hopkins University
BEVERLY, STEPHEN, Harvard Medical School
BLOOM, BARRY, Albert Einstein College of Medicine
BROWN, KIM H., University of Iowa
BURAKOFF, STEVEN J., Harvard Medical School
CARTER, RICHARD, NIAID/NIH
CANTOR, CHARLES, Columbia University
CERAMI, ANTHONY, Rockefeller University
CHEEVER, ALLEN, NIAID/NIH
CLEVELAND, DON, Johns Hopkins School of Medicine
DALTON, JOHN, Johns Hopkins School of Medicine
DAVIS, MARK, Stanford University
DINTZIS, HOWARD, Johns Hopkins School of Medicine
DONELSON, JOHN, University of Iowa
DWYER, DENNIS, M., NIH
GEARHART, PATRICIA, Johns Hopkins School of Medicine
HART, GERALD W., Johns Hopkins School of Medicine
HOWARD, RUSSELL, NIAID/NIH
HOWARD, JAMES, Wellcome Laboratories, U.K.
JAMES, STEPHANIE, George Washington School of Medicine
JOINER, KEITH, NIAID/NIH
KNOPF, PAUL, Brown University
KRAKOW, JESSICA L., Johns Hopkins School of Medicine
LENARDO, MICHAEL, University of Iowa
MCGUIRE, MARIELENA VALEZ, NIAID/NIH
MOSS, BERNARD, NIAID/NIH
MARSDEN, PHILIP, Federal University of Brasilia, Brazil
NASH, THEODORE, NIAID/NIH
NEVE, FRANKLIN A., NIH
NELSON, GEORGE, University of Liverpool, U.K.
NUSSENZWEIG, VICTOR, New York University School of Medicine
PFEFFERKORN, ELMER, Dartmouth Medical School
VAN DER PLOEG, LEX, Columbia University
RAVDIN, JONATHAN, University of Virginia School of Medicine
SACKS, DAVID, NIAID/NIH
SCOTT, PHILLIP, NIAID/NIH
SHER, KATHY, Columbia University
SHEVACH, ETHAN, NIH
SNARY, DAVID, Wellcome Laboratories, U.K.
SPIELMAN, ANDREW, Harvard Medical School
STRAND, METTE, Johns Hopkins School of Medicine
TURNER, MERVYN J., Merck Sharp & Dohme
ULLMAN, BUDDY, University of Kentucky Medical Center
WANG, CHING C., University of California
WARREN, KENNETH, Rockefeller Foundation
WASSOM, DAVID L., Cornell University
WARD, DAVID, Yale University

Students

ANDREWS, NORMA W., New York University
ARGUELLO, CARLOS LOPEZ, National Polytechnic Institute, Mexico

Barral, Aldina, M. P., Federal University of Bahia, Brasil
Bennett, Karen L., Carnegie Institution
Burns, James M., Jr., Hahnemann University
Cordova, Jose L., Boston University
Doering, Tamara L., Johns Hopkins School of Medicine
Febbraio, Maria, Cornell University Medical School
Foley, Michael, University of Dundee, U.K.
Johnson, Rollin Breese, Cambridge University, U.K.
Lafaille, Juan Jose, Instituto de Quimica, Brasil
Limo, Moses Kiprop, International Laboratory for Research on Animal Diseases, Kenya
Mogyoros, Myriam K., Weizmann Institute of Science, Israel
Osotimehin, Babatunde, University of Ibadan, Nigeria
Woollett, Gillian, University of Edinburgh, U.K.
Zentella-Dehesa, Alejandro, Rockefeller University

Cellular Neurobiology in the Leech

Course director

Nicholls, John, University of Basel, Switzerland

Other faculty, staff, and lecturers

Calabrese, Ronald, Harvard University
Friesen, W. Otto, University of Virginia
Kristan, William, University of California, San Diego
Macagno, Eduardo, Columbia University
Muller, Kenneth, University of Miami Medical School
Payton, Brian, Memorial University of Newfoundland, Canada
Ross, William, New York Medical College
Salzberg, Brian M., University of Pennsylvania
Stent, Gunther, University of California, Berkeley
Thompson, Wesley J., University of Texas
Weisblat, David A., University of California
Wilson, Darcy B., Scripps Clinic and Research Institute
Zipser, Birgit, NIH

Students

Acklin, Susan, University of Basel, Switzerland
Hyson, Richard L., University of Colorado
Law, Margaret I., University of California, Berkeley
Lev-Ram, Varda, Weizmann Institute of Science, Israel
Marom, Shimon, Israel Institute of Technology, Israel
Midtgaard, Jens, Kobenhavns University, Denmark
Moneta, Maria Eugenia, University of Chile, Chile
Pessin, Melissa S., Johns Hopkins School of Medicine
Wilhere, George F., Northwestern University
Wilson, Gisela F., University of Wisconsin

Embryology: A Modern Course in Developmental Biology

Course directors

Brandhorst, Bruce, McGill University, Canada
Jeffery, William, University of Texas

Other faculty, staff, and lecturers

Axel, Richard, Columbia University
Bates, William, University of Texas
Beach, Rebecca, University of Texas
Borisy, Gary G., University of Wisconsin
Brinkley, B. R., University of Alabama
Chikaraishi, Dona, Tufts University
Condeelis, John, Albert Einstein College of Medicine
Crain, William, Worcester Foundation of Experimental Biology
Crain, Caleb, Worcester Foundation of Experimental Biology
Dawid, Igor, NIH
Dohmen, M. R., University of Utrecht, Netherlands
Dworkin, Mark, Columbia University
Dworkin-Rostl, Eva, Columbia University
Falkenthal, Scott, Ohio State University
Firtel, Richard A., University of California
Gehring, Walter, University of Basel, Switzerland
Gerhart, John C., University of California
Goldhammer, David, Ohio State University
Goudeau, Henri, Cen Saclay, France
Goudeau, Marie, University of Marie Curie, France
Gurney, M., University of Chicago
Gurdan, John, Cambridge University, U.K.
Harris, Albert K., University of North Carolina
Hille, Merrill B., University of Washington
Holland, Linda, Scripps Clinic and Research Institute
Jackle, Herbert, Max Planck Institute, FRG
Jacobson, Antone, University of Texas
Jaffe, Laurinda, University of Connecticut
Kado, Raymond, Centre National/Recherche Scientifique, France
Kline, Douglas, University of California
Lapuk, Seth, University of Connecticut
Lengyel, Judith, University of California
Levine, M., Columbia University
Maul, Gerd, The Wistar Institute
Maxson, Robert E., Jr., University of Southern California
Myles, Diane, University of Connecticut
Morrow, Laura, University of Texas
Newman, Stuart A., Cornell University Medical School
Olins, Josh, Earlham College
Ordahl, Charles, University of California
Rankin, Mary Ann, University of Texas
Rubin, L., Rockefeller University
Sanger, Joseph, University of Pennsylvania
Sater, Amy, University of Texas
Schatten, Gerald, Florida State University
Schatten, Heidi, Florida State University
Schultz, Gilbert A., University of Calgary, Canada
Shur, Barry, University of Texas
Shrutkowski, Anthony, Columbia University
Smith, L. Dennis, Purdue University
Szellosi, Daniel, Stazione Centrale Physiologie Animale, France
Grant-Takeda, Nancy J., University of Strasbourg, France
Turner, Paul, University of Connecticut Health Center
Uzman, Akif, University of Texas

Students

Allen, Philip G., Jr.**, Harvard University
Basolo, Alexandra*, University of Texas
Beis, Androniki, University of Thessalonki, Greece
Blair, Seth S.*, University of Washington
Brault, Elisabeth C.*, Centre National/Recherche Scientifique, France
Chisholm, Julia Clare*, University of Cambridge, U.K.
Cole, Eric S., University of Iowa
Coffe, Gerard*, University of Paris, France
Dike, Laura E.*, Boston University
Dube, Francois, Stanford University
Dubin, Robert A., City University of New York
Epstein, Helen*, Columbia University
Farquharson, Andrew*, University of Glasgow, U.K.
Golsteyn, Roy M.*, University of Calgary, Canada
Hafner, Mathias, German Cancer Research Center, FRG
Howard, Kenneth Ramsey, University of London, U.K.
Iglesias, Ana, University of Connecticut Health Center
Kao, Kenneth R.**, University of Toronto, Canada
Miotta, Karen A., University of Colorado
Moury, John David*, University of Texas
Ransick, Andrew J.*, University of Texas
Rayson, Thomas C., University of Hawaii
Speksnijder, Johanna E.*, University of Utrecht, Netherlands
Swalla, Billie J.**, University of Iowa
Telfer, Abby*, Harvard University
Turner, Paul R.**, University of Connecticut Health Center
Welch, Jeffrey E.*, University of North Carolina

MARINE ECOLOGY

Course director

Frank, Peter W., University of Oregon

Other faculty, staff, and lecturers

Alberte, Randall, University of Chicago
Bert, Theresa M., Yale University
Buss, Leo, Yale University
Carlton, James, Williams College, Mystic Seaport
Caraco, Nina, Mary Flager Cary Arboretum, New York
Caswell, Hal, Woods Hole Oceanographic Institution
Connell, Joseph, University of California
Cole, Jon, Mary Flager Cary Arboretum, New York
Foreman, Kenneth, Marine Biological Laboratory
Frost, B. W., University of Washington
Gallagher, Eugene, University of Massachusetts
Giblin, Ann, Marine Biological Laboratory
Grassle, J. Frederick, Woods Hole Oceanographic Institution
Grassle, Judith, Marine Biological Laboratory
Harbison, G. R., Woods Hole Oceanographic Institution
Hobbie, John, Marine Biological Laboratory
Howarth, Robert, Marine Biological Laboratory

* Post-course Participants
** Advanced Research Training Program Participants

Jannasch, Holger, Woods Hole Oceanographic Institution
Jefferies, Robert L., University of Toronto, Canada
Kelly, Jack, Cornell University
Koehl, Mimi, University of California, Berkley
Mann, Kenneth H., Bedford Institute of Oceanography, Canada
Odum, William, University of Virginia
Osman, Richard, Academy of Natural Sciences of Philadelphia
Peterson, C. H., University of North Carolina
Posey, Martin, University of Oregon
Rhoads, Donald, Yale University
Rietsma, Carol, SUNY, New Paltz
Scheltema, Rudolph, Woods Hole Oceanographic Institution
Sebens, Kenneth, Northeastern University
Smith, Robert, University of Chicago
Stoecker, Diane, Woods Hole Oceanographic Institution
Valiela, Ivan, Boston University
Vittor, Barry, B. Vittor and Associates
Welshmeyer, Nicholas, Harvard University
Woodwell, George, Woods Hole Research Center

Students

Avol, Sandra L.*, Yale University School of Medicine
Barnett, Bruce A.*, North Dakota University
Bocca, Adrian Horacio*, Universidad Nacional del Comahue, Argentina
Burgos, Guillermo E.*, National Institute/Fishery Research, Argentina
Fee, Elisabeth J.*, Ohio State University
Hohman, William L., University of Minnesota
Iribarne, Oscar Osvaldo*, Universidad Nacional del Comahue, Argentina
Martinez, Daniel Eduardo*, Universidad Nacional Bahia Blanca, Argentina
Morek, Michele M., Brescia College
Mulsow, Sandor G.*, Universidad Austral de Chile, Chile
Ray, Andrea J.*, University of Chicago
Renzi, Marta Alicia*, National Institute/Fishery Research, Argentina
Scott, Dillon*, University of Massachusetts
Tresierra-Aguilar, Alvaro*, National University of Trujillo, Peru
Verazay, Guillermo A., National Institute/Fishery Research, Argentina
Zea, Sven E.*, University of Texas

MICROBIOLOGY: MOLECULAR ASPECTS OF CELLULAR DIVERSITY

Course directors

Greenberg, Peter, Cornell University
Wolfe, Ralph, University of Illinois

Other faculty, staff, and lecturers

Blakesmore, Richard, University of New Hampshire
Dilling, Waltraud, University of Konstanz, FRG
Frankel, Richard, Massachusetts Institute of Technology
Harrigan, June, Marine Biological Laboratory
Hartzell, Patricia, University of Illinois
Harwood, Caroline, Cornell University
Kaplan, Heidi, Cornell University
Leigh, John, Massachusetts Institute of Technology
Leadbetter, Jared, Marine Biological Laboratory
Schink, Bernard, University of Konstanz, FRG

* Post-course Participants

Students

Bobik, Thomas A., University of Illinois
Bryden, Cynthia G., Boston University Marine Program
Coutinho, John B., Queen's University, Canada
Damerval, Thierry, Institute Pasteur, France
Dunlap, Paul V., Cornell University
Gray, Kendall M., University of California
Lewitus, Alan J., Woods Hole Oceanographic Institution
Marinelarena, Alejandro J., Universidad Nacional de la Plata, Argentina
Martin, Michael M., University of Michigan
O'Brien, Wendy A., Dana-Farber Cancer Institute
Pascual-Spada, Maria Mercedes, Universidad de Santa Ursula, Argentina
Pratt, Charles W., University of Illinois
Rouviere, Pierre E., University of Illinois
Schwarzenbach, Rene P., Swiss Federal Institute for Water Resources, Switzerland
Smith, Clifford Mark, Harvard University
Stanton, Alicia M., SUNY, Geneseo
Summers, Lisa Carol, Swarthmore College
Trent, Jonathan D., Scripps Institution of Oceanography
Warshaw, Jane E., University of Massachusetts
Zeyer, Josef A., Swiss Federal Institute for Water Resources, Switzerland

MOLECULAR AND CELLULAR IMMUNOLOGY

Course director

Reinisch, Carol, Tufts University School of Veterinary Medicine

Other faculty, staff, and lecturers

Acuto, Oreste, Dana-Farber Cancer Institute
Alper, Chester, Center for Blood Research
Barbosa, James, Dana-Farber Cancer Institute
Beller, David, Harvard Medical School
Bevan, Michael, Scripps Clinic and Research Institute
Boss, Jerry, Harvard University
Capra, J. Donald, University of Texas
Cebra, John J., University of Pennsylvania
Datta, Syamal, Tufts University
Eisenbarth, George S., Joslin Diabetes Clinic
Fink, Pamela, University of California Cancer Center, San Diego
Groopman, Jerome E., Deaconess Hospital
Hochman, Paula, Tufts University
Hogg, Nancy, Imperial Cancer Research Fund, England, U.K.
Joiner, Keith, NIAID/NIH
Kincade, Paul W., Oklahoma Medical Research Foundation
Leskowitz, Sidney, Tufts University
McClusky, Robert, Massachusetts General Hospital
Mosier, Don, Medical Biological Institute
Nadler, Lee M., Dana-Farber Cancer Institute
Parker, Kenneth, Harvard University
Parker, David C., University of Massachusetts Medical School
Richard, Frank, Yale School of Medicine
Rocklin, Ross, Tufts New England Medical Center
Schlossman, Stuart, Dana-Farber Cancer Institute
Schwartz, Robert, Tufts New England Medical Center
Sprent, Jonathan, Scripps Clinic and Research Institute

Strom, Terry, Massachusetts General Hospital
Sunshine, Geoffrey H., Tufts University
Theoharides, Theoharis, Tufts University
Wall, Randolph, University of California, Los Angeles
Wilson, Darcy B., Medical Biological Institute

Students

Bakst, Murray R., U. S. Department of Agriculture
Balazs, Tibor, U. S. Food and Drug Administration
Cinquina, Carmela L., West Chester University
Conway, Carolyn M., Virginia Commonwealth University
Graze, Kathleen K., New York State Psychiatric Institute
de Holl, John David, Washington and Lee University
Jacobs, Jerome B., St. Vincent Hospital
Kommineni, Choudari, Mobil Oil Corporation
Ligas, James R., University of Connecticut
Mamuya, Wilfred, Boston University
McCarthy, James G., University of Connecticut
McFarland, Walter G., University of Louisville
Meydani, Simin N., Tufts University
Miranda, Maria T., University of Puerto Rico
Phillips, Fredric J., John F. Kennedy University
Rao, Potu N., University of Texas
Saba, Thomas, Albany Medical College
Smith, Jessica L., Ohio University
Troesch, Claudia D., University of Southern California

Neural Systems and Behavior

Course directors

Carew, Thomas, Yale University
Kelley, Darcy, Columbia University

Other faculty, staff, and lecturers

Bass, Andrew, Cornell University
Burd, Gail, Rockefeller University
Byrne, John, University of Texas Medical School
Calabrese, Ronald, Harvard University
Grober, Matthew, University of California, Los Angeles
Haerter, Ursula, Cornell University
Harris-Warrick, Ronald, Cornell University
Hoskins, Sally, Columbia University
Johnson, Bruce, Boston University
Karten, Harvey, SUNY, Stony Brook
Macagno, Eduardo, Columbia University
Marder, Eve, Brandeis University
Marler, Peter, Rockefeller University
Marchaterre, Martin, Marine Biological Laboratory
McRobert, Scott, Temple University
Menzel, Randolph, Free University of Berlin, FRG
Peinado, Alex, Columbia University
Rusak, Benjamin, Dalhousie University, Canada
Schafer, Sabine, Free University of Berlin, FRG
Segil, Neil, Columbia University
Simpson, Blair, Rockefeller University
Thompson, Richard, Stanford University

TOMPKINS, LAURIE, Temple University
TOBIAS, MARTHA, Columbia University
WALSH, JOHN, University of Texas Medical School
WEEKS, JANIS, University of California
ZIPSER, BIRGIT, NIH

Students

BAADER, ANDREAS, University of Berlin, FRG
CRONER, LISA J., Duke University
DUBAS, FRANCOISE, University of Texas
FEIGENBAUM, JANET D., Stanford University
FRIEDMAN, ROBERT M., C. B. Wilson Center
HODGSON, TRACY M., Wayne State University
INGRAM, DONNA A., University of Texas
KATAYAMA, AKIKO, University of Hawaii
LOHMANN, KENNETH J., University of Washington
MARCUS, EMILIE A., Yale University
MCPHERSON, DUANE, University of Texas Medical Branch
MONTEMAYOR, MICHELLE E., University of Illinois
PELUFFO, LUCAS, University of California, San Diego
READ, HEATHER L., University of Texas
REIMER, KATRIN, University of Munich, FRG
RIOULT-PEDOTTI, MARC GUY, Swiss Federal Institute of Technology, Switzerland
RIOULT-PEDOTTI, MENGIA SERAINA, University of Zurich, Switzerland
SCHARFF, CONSTANCE, Rockefeller University
SCHOLZ, KENNETH P., University of Texas
STENGL, MONIKA, Columbia University

NEUROBIOLOGY

Course director

KARLIN, ARTHUR, Columbia University

Other faculty, staff, and lecturers

ADAMS, PAUL, SUNY, Stony Brook
ANDREWS, BRIAN, NINCDS/NIH
ANDERSON, DAVID, Columbia University
BAKALYAR, HEATHER, William Smith College
BLUM, MARIANNE, Columbia University
BRETT, ROGER, SUNY, Stony Brook
FISHBACH, GERALD, Washington University School of Medicine
GREENGARD, PAUL, Rockefeller University
GURNEY, ALISON, California Institute of Technology
HALL, LINDA, Albert Einstein College of Medicine
HEUSER, JOHN, Washington University School of Medicine
HESS, PETER, Yale University
HUSE, WILLIAM, Yale School of Medicine
HUDSON, RICHARD, University of Toledo, Canada
INOUE, TED, Cornell University
JONES, STEVEN, SUNY, Stony Brook
KAO, PETER, Columbia University
KANDEL, ERIC, Columbia University
LANDIS, DENNIS, Massachusetts General Hospital
LANDIS, STORY, Harvard Medical School
LEVITAN, IRWIN, Brandeis University
LESTER, HENRY, California Institute of Technology

LISMAN, JOHN, Brandeis University
MARDER, EVE, Brandeis University
MARLER, JENNIFER, Yale University
MATSUMOTO, STEVEN, Harvard Medical School
MILLER, CHRISTOPHER, Brandeis University
RAVIOLA, ELIO, Harvard Medical School
REESE, THOMAS, NINCDS/NIH/Marine Biological Laboratory
REEDY, MICHAEL, Duke University Medical School
RICHARDS, FREDERIC M., Yale University
ROBERTS, JAMES, Columbia University
ROWLAND, LEWIS, Columbia University
SIGWORTH, FRED, Yale University School of Medicine
SILMAN, ISRAEL, Weizmann Institute, Israel
SPUDICH, JOHN, Albert Einstein College of Medicine

Students

BALICE-GORDON, RITA J., University of Texas
BREZINA, VLADIMIR, University of California, Los Angeles
FALLS, DOUGLAS L., Ellis Hospital
ITAGAKI, HARUHIKO, University of Alabama
IVENS, INGEBORG, Ruhr-Universitat Bochum, FRG
KOROSHETZ, WALTER JOSEPH, Harvard Medical School
MONTAGUE, P. READ, University of Alabama, Birmingham
OWENS, JESSE L., University of Alaska
REGAN, LAURA J., Harvard University
RICH, MARK M., Washington University
SAEZ, JUAN, Albert Einstein College of Medicine
SUTTON, FEDORA, Howard University

PHYSIOLOGY: CELL AND MOLECULAR BIOLOGY

Course director

GOLDMAN, ROBERT, Northwestern University

Other faculty, staff, and lecturers

ALBRECHT-BUEHLER, G., Northwestern University
BENDER, WELCOME, Harvard Medical School
BLOOM, KERRY, University of North Carolina
CANDE, W. ZACHEUS, University of California
CHISHOLM, REX, Northwestern University
DUTCHER, SUSAN, University of Colorado
EARNSHAW, WILLIAM, Johns Hopkins University
FUKUI, YOSHIO, Osaka University, Japan
GETHING, MARY JANE, Cold Spring Harbor Laboratory
GLASER, LUIS, Washington University School of Medicine
GREEN, KATHLEEN, Northwestern University
HUBBARD, ANN, Johns Hopkins University
HYAMS, JEREMY, University College, U.K.
JAMIESON, JAMES, Yale University School of Medicine
JONES, JONATHAN, Northwestern University
KABACK, D., University of New Jersey
LEINWAND, LESLIE, Albert Einstein Medical School
LUCA, FRANK, Worcester Foundation of Experimental Biology
MAYRAND, SANDRA, Worcester Foundation of Experimental Biology
MCGOVERN, KAREN J., Dana-Farber Cancer Institute
MCNALLY, ELIZABETH, Albert Einstein Medical School

Murray, Andrew, Massachusetts General Hospital
Pederson, Thoru, Worcester Foundation of Experimental Biology
Roeder, Robert G., Rockefeller University
Rushforth, Alice, Earlham College
Schwartz, Lawrence M., University of North Carolina
Soll, David, University of Iowa
Spudich, James, Stanford University
Steinert, Peter, NIH
Utku, Shermin, Earlham College
Vallee, Richard, Worcester Foundation of Experimental Biology
Weben, Eric D., Mayo Foundation
Witman, George, Worcester Foundation of Experimental Biology
Yeh, Elaine, CIBA-GEIGY

Students

Barbe, Mary F., Wake Forest University
Barish, Michael E., University of California, Los Angeles
Blackmon, Ronald H., Howard University
Bockman, Richard S., Cornell University Medical College
Byers, Timothy J., Harvard University
Campbell, Colin R., Boston University School of Medicine
Cassimeris, Lynne U., University of North Carolina
Chen, Tung-Ling L., University of Maryland
Danowski, Barbara A., University of North Carolina
Edson, Kathryn J., University of Minnesota
Freeman, Mark E., University of Virginia
Goodwin, Elizabeth, Brandeis University
Haluska, Frank G., University of Pennsylvania
Hanna, Maya, Harvard University
Harmon, Gary L., Howard University
Hunnicutt, Gary R., University of Texas Health Science Center
Lacey, Merri Lynn, University of California, Riverside
Lans, Deborah A., University of California, Berkeley
Lenart, Thomas D., Johns Hopkins University
Noorily, Stuart W., Boston University
Obar, Robert A., Boston University
O'Halloran, Theresa J., University of North Carolina
Pines, Jonathon N., Cambridge University, U.K.
Pret, Anne-Marie, Wesleyan University
Schumaker, Karen S., University of Maryland
Troesch, Claudia D., University of Southern California
Tuttle, Rebecca, Purdue University
Vikstrom, Karen Lynn, University of Michigan
Walther, Zenta, Yale University
Wang, Gordon V. L., University of Hawaii
Warner, Cecilia A., Northwestern University
Weston, Wayde M., University of Pennsylvania
Wu, Bei-Yue, Wayne State University
Wu, Julian K., Tufts University
Yabowitz, Rachel, University of Miami

SPRING

Biophysics of Neural Function

Course director
Alkon, Daniel L., NINCDS/NIH, Marine Biological Laboratory

Other faculty, staff, and lecturers

Brodwick, Malcolm, University of Texas
Brightman, Milton, NINCDS/NIH
Charlton, Milton, University of Toronto, Canada
Clapham, David, Harvard Medical School
Connor, John, Bell Laboratories
DeFelice, Louis, Emory University School of Medicine
DeLorenzo, Robert, Yale University School of Medicine
Dionne, Vincent E., University of California, San Diego
Finkelstein, Alan, Albert Einstein College of Medicine
Foreman, Robin, Marine Biological Laboratory
French, Robert, University of Maryland School of Medicine
Gilbert, Charles, Rockefeller University
Govind, C. K., University of Toronto, Canada
Gruol, Donna L., Research Institute of Scripps Clinic
Hochberger, Phillip, Bell Laboratories
Johnson, Bruce, Boston University
Kaplan, Ehud, Rockefeller University
Lecar, Harold, NIH
Matsumoto, Hiro, Purdue University
McDermott, Amy, NIH
Morris, Catherine, University of Ottawa, Canada
Moran, Nava, NINCDS/NIH
Palotta, Barry, University of North Carolina
Pappas, George, University of Illinois Medical Center
Pollard, Harvey, NIADDKD/NIH
Rasmussen, Howard, Yale University School of Medicine
Reese, Thomas, NINCDS/NIH/Marine Biological Laboratory
Rojas, Eduardo, NIADDKD/NIH
Schnapp, Bruce, Marine Biological Laboratory
Szuts, Ete, Marine Biological Laboratory
Weiss, Thomas F., Massachusetts Institute of Technology
Yeo, Christopher, Medical Research Council, U.K.
Zbicz, Kerry, NIAAA/NIH

Students

Brew, Helen M., University College, London, U.K.
Chandramani, Nina, Syracuse University
Chen, Chong, University of Rhode Island
Galeno, Theresa M., New York State Department of Health
Garg, Ajay P., Albert Einstein College of Medicine
Maguire, Gregory W., University of Miami
Mazzanti, Michele, Emory University School of Medicine
McClintock, Timothy S., University of Florida
Owens, Jesse L., University of Alaska
Park, Susanna S., Yale University
Rice, Peter J., West Virginia University Medical Center
Satter, Ruth L., University of Connecticut
Silva, Nancy L., Yale University School of Medicine
Srebro, Bolek, University of Bergen, Norway
Tahmoush, Albert J., Hahnemann University
Zufall, Frank, Institute of Animal Physiology, FRG

SHORT COURSES

BASIC IMMUNOCYTOCHEMICAL TECHNIQUES IN TISSUE SECTIONS AND WHOLE MOUNTS

September 30–October 5, 1985

Course directors

BELTZ, BARBARA, Harvard Medical School
BURD, GAIL D., Rockefeller University

Course assistants

KOBIERSKI, LINDA, Harvard Medical School
O'LOUGHLIN, BARBARA, Rockefeller University

Students

BEVAN, JOHN A., University of Vermont
CALLARD, IAN P., Boston University
CHIKARAISHI, DONA, Tufts University School of Medicine
COPELAND, JONATHAN, Swarthmore College
HAYASHI, JON H., University of North Carolina
JENSEN, KARL, U. S. Environmental Protection Agency
MEKEEL, HAROLD E., Harvard Medical School
OAKLEY, BRUCE, University of Michigan
OLAND, LYNNE A., Georgetown University
STUART, ANN E., University of North Carolina
TALAMO, BARBARA R., Tufts Medical School
TIEMAN, SUZANNAH BLISS, SUNY, Albany
YAHR, PAULINE, University of California, Irvine

MARICULTURE: CULTURE OF MARINE INVERTEBRATES FOR RESEARCH PURPOSES

May 19–25, 1985

Course director

CARL BERG, JR., Marine Biological Laboratory

Other faculty and assistants

ADEY, WALTER, Smithsonian Institute
BOWER, CAROL, Eastern Connecticut State University
CAPO, THOMAS, Marine Biological Laboratory
CAPUZZO, JUDITH, Woods Hole Oceanographic Institution
DOYLE, ROGER, Dalhousie University, Canada
GARIBALDI, LOUIS, New York Aquarium
GUILLARD, BOB, Bigelow Laboratories
HANLON, ROGER, University of Texas
HARRIGAN, JUNE, NIH/Marine Biological Laboratory
LANGDON, CHRISTOPHER, University of Delaware
TURNER, DAVID, Eastern Connecticut State University

Students

ABRANT, RONALD J., Loyola University
BACHAND, ROBERT G., Long Island Sound Task Force Oceanic Society
GARFIELD, NINA H., Western Psychiatric Institute
GIRALDO, JORGE A., Washington University
HAWES, ROBERT O., University of Maine
HERZIG, CHRIS, Occidental College
HINES, JOHN L., Massachusetts General Hospital
KOCHANE, IDA, Newtonville, MA
KUTZ, STEVEN L., George Mason University

STABILE, JOSEPH, City College, CUNY
WARD, STEPHEN H., U. S. Environmental Protection Agency

THREE INTEGRATED SHORT COURSES IN QUANTITATIVE IMAGE AND SIGNAL ANALYSIS FOR BIOLOGISTS

December 9–19, 1985

Faculty

BUSCHMANN, ROBERT J., VA Medical Center, Chicago
HASELGROVE, JOHN C., University of Pennsylvania
JONES, JUDSON P., University of Pennsylvania
PALMER, LARRY A., University of Pennsylvania
PEACHEY, LEE D., University of Pennsylvania

Students

ARIANO, MARJORIE, University of Vermont College of Medicine
ASTION, MICHAEL, University of Pennsylvania School of Medicine
BEUERMAN, ROGER, Louisiana State University Eye Center
BOSCH, ELIZABETH, University of California, Los Angeles
BRAVO, MARY, Northwestern University
CROPPER, ELIZABETH, Columbia University
ECKERT, BARRY S., SUNY, Buffalo
EISENMAN, LEONARD M., Jefferson Medical College
FAMIGLIETTI, EDWARD V., JR., Wayne State University
FRANZ, THOMAS P., Procter and Gamble Co.
FRIEDMAN, MARC M., Georgetown University
GRADY, PATRICIA A., University of Maryland School of Medicine
HEATH, JULIAN P., University of Pennsylvania
JACOBS, MYRON S., New York University Dental Center
KETTEN, DARLENE R., Massachusetts Eye and Ear Infirmary
LAVIA, LYNN A., University of Kansas School of Medicine
LUCZKA, CAROL JEAN, Cornell University
MCCAULEY, BRIGID, University of Missouri, St. Louis
MCEACHRON, DONALD LYNN, Drexel University
MILLARD, PAUL J., Cornell University
MOHR, CHARLES, Cornell University
MOSES, RANDY L., Louisiana State University Medical Center
NELSON, GINA M., University of Colorado, Denver
PAINE, PHILIP L., Michigan Cancer Foundation
PARK, JANIE C., Florida Institute of Technology
PECK, CAROL K., University of Missouri, St. Louis
POOLE, MAX, East Carolina University
PREVETTE, DAVID M., Bowman Gray School of Medicine
ROYE, DAVID B., University of North Carolina, Wilmington
SENS, MARY ANN, Medical University of South Carolina
SPOSITO, NADINE M., SUNY Stony Brook
THORNBURG, KENT L., Oregon Health Sciences University
WHEELER, DAVID A., Brandeis University
YANO, BARRY L., Dow Chemical, U.S.A.

X. RESEARCH AND TRAINING PROGRAMS

SUMMER

PRINCIPAL INVESTIGATORS

ALLEN, ROBERT DAY, Dartmouth College
ANDERSON, WINSTON A., Howard University

Armstrong, Clay M., University of Pennsylvania
Armstrong, Peter B., University of California, Davis
Augustine, George, University of Southern California
Bacigalupo, Juan, University of Chile, Chile
Barlow, Robert B., Syracuse University
Barry, Daniel, University of Michigan
Barry, Susan R., University of Michigan
Beauge, Luis Alberto, Instituto de Investigacion Medica, Argentina
Bennett, Michael V. L., Albert Einstein College of Medicine
Bezanilla, Francisco, University of California, Los Angeles
Bodznick, David, Wesleyan University
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Reese, Thomas S.

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Cheng, Toni P. O.
Evenden, Phyllis
Fels, Gregor, Max Planck Institut, FRG
Frokjaer-Jensen, Jorgen, University of Copenhagen, Denmark
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Khan, Shahid, Marine Biological Laboratory
Michaud, Jayne
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Reese, Barbara F.
Sheetz, Michael P., Washington University
Schnapp, Bruce J.
Vale, Ronald D.
Walrond, John P.
Wisgirda, Mary, Marine Biological Laboratory
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National Vibrating Probe Facility

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Ettensohn, Charles, Duke University
Fluck, Richard, Franklin & Marshall College
Katz, Uri, Israel Institute of Technology, Haifa, Israel
Kunkel, Joseph, University of Massachusetts
Levy, Simon, Boston University
Machen, Terence, University of California, Los Angeles
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Qui, Ting-Hu, Xiamen Fisheries College, Fujian, China
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Semino, Suzanne J.
Shaver, Gaius R.
Steudler, Paul A.
Stone, Thomas
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White, David
Yandow, Timothy

Trainees

Rudnick, David, University of Rhode Island

Visiting investigator

Luther, George, Kean College of New Jersey

XI. HONORS

Friday Evening Lectures

Allen, Robert D., Dartmouth College, 28 June, *"Microtubules, Motility, and Cytoplasmic Transport"*

Hildebrand, John G., Columbia University, 5 July, Lang Lecture, *"Explorations of a Miniature Brain"*

Leder, Philip, Harvard Medical School, 12 July, *"Misplacing Genes: Genetic Engineering and the Cancer Problem"*

Raff, Martin, University College, London, 18, 19 July, Forbes Lectures, *"An Antibody and Cell Culture Approach to Mammalian Neurodevelopment: I. Cell-Cell Interactions in the Developing Peripheral Nervous System; II. Cell Lineages and Cell Differentiation in the Developing Central Nervous System"*

Edelman, Gerald, The Rockefeller University, 26 July, *"Cell Adhesion Molecules and the Regulation of Animal Form"*

Gehring, Walter, Biozentrum, University of Basel, 2 August, *"Homeotic Genes and the Control of Development"*

Benacerraf, Baruj, Harvard Medical School, 9 August, *"Immunology or an Insight into Nature's Identity System"*

Allen, Garland E., Washington University, 16 August, *"T. H. Morgan and the MBL: A Tale of Embryos and Genes"*

Mastroianni, Luigi, Jr., University of Pennsylvania, 23 August, C. Lalor Burdick Lecture, *"Human* in vitro *Fertilization: The New Frontier in Gamete Physiology"*

Associates' Lecture

Gross, Paul R., Marine Biological Laboratory, 17 August, *"Builders and Science of the MBL"*

Frederick B. Bang Fellowship

Marcum, James A., Massachusetts Institute of Technology

Charles Ulrick and Josephine W. Bay Foundation Fellowship

Smolowitz, Roxanna, Marine Biological Laboratory

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Hough, Rose A.
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EVOLUTION AND FUNCTION OF STRUCTURALLY DIVERSE SUBUNITS IN THE RESPIRATORY PROTEIN HEMOCYANIN FROM ARTHROPODS

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ABSTRACT

Native aggregation level and subunit composition of the hemocyanins from 86 adult chelicerates and crustaceans, and from the larval stages of 2 crabs, were analyzed by means of electron microscopy, polyacrylamide electrophoresis, immuno blotting, and crossed immunoelectrophoresis, supported by a variety of preparative separation techniques. The up to eight immunologically discernible subunit types were interspecifically correlated, classified, and evolution lines derived. Phylogenetic consequences are discussed, and are particularly aggravating in spiders.

A single subunit suffices for the formation of hexamers (1 × 6). In the architecture of higher-ordered hemocyanins, the various subunits act as building-blocks of distinct specification. This was studied in 2 × 6 molecules from a hunting spider and several crustaceans, and in 4 × 6 hemocyanin from a tarantula. The various subunits are present in constant proportions. The total set is required to reorganize the original aggregate from subunit mixtures. Stable oligomeric segments of native hemocyanin particles revealed the gross distribution of the diverse subunits. Immuno electron microscopy of the native hemocyanins decorated with monospecific Fab fragments showed the exact topographic position of each subunit type, and detailed models of the quaternary structure could be derived.

The oxygen binding function of 4 × 6 hemocyanin from the tarantula *Eurypelma californicum* is excessively modulated by subunit interaction phenomena. We measured native, reassembled, and mercury-blocked 4 × 6-mers, oligomeric segments, single subunits, and reassembled 4 × 6-mers with one subunit type chemically modified. The spatial range of allosteric interaction, and specific contributions of the diverse subunits are outlined.

INTRODUCTION

Hemocyanin, a blue copper-protein, functions as an oxygen carrier in the blood of arachnids, horseshoe crabs, crustaceans, and centipedes. Another hemocyanin occurs in inkfishes, chitons, many snails, and some primitive bivalves; however, despite certain similarities, molluscan hemocyanin differs decisively from arthropod hemocyanin in its main structural features, and therefore will not be discussed here. Most certainly, both pigments have evolved independently from tyrosinase, an ancestral precursor. Arthropod hemocyanins are multi-subunit proteins with a molecular mass of about 75,000 per polypeptide chain. The polypeptides—or subunits—are arranged as cubic hexamers (1 × 6), or multiples of hexamers (2 × 6, 4 × 6, 6 × 6, 8 × 6); the native aggregation level is species-specific. (Ghiretti, 1968; Van Holde and van Bruggen, 1971; Van Holde and Miller, 1982; van Bruggen *et al.*, 1982; Ellerton *et al.*, 1983;

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Huber and Lerch, 1986; Mangum *et al.,* 1985; Morse *et al.,* 1986). In molecular mass, the 8 × 6-mer (48 subunits) exceeds human hemoglobin 50-fold, and measures about 25 nanometers across, corresponding to the size of a ribosome. The three-dimensional structure of the basic hexamer recently was elucidated in detail (Gaykema *et al.,* 1984). All hemocyanin aggregates are clearly visible in the electron microscope (Fig. 3); the arrangement of hexamers in the higher-ordered molecules, as shown in Figure 1, was partially derived by computer image analysis (Van Heel and Frank, 1981; Bijlholt *et al.,* 1982). Hemocyanin is not incorporated in cells, due to physical problems (Mangum, 1985), but floats freely in the blood in concentrations up to 120 mg/ml (scorpions), meaning that it fills up to $^1/_{10}$ of the hemolymph space; the average distance from one molecule to the next equals its own diameter. Arthropod hemocyanin particles dissociate at alkaline pH into their subunits, which are capable still of reversibly binding oxygen. By dialysis of the obtained subunit mixtures against neutral pH, a self-assembly at least of hexamers, and frequently also of higher-ordered molecules occurs.

Each subunit is a delicate structure of around 10,000 atoms: 620 amino acids, which form three domains containing the binuclear copper site, 20 alpha helices, and a large seven-stranded beta barrel (Gaykema *et al.,* 1984; Linzen *et al.,* 1985). At first glance this appears to be a considerable luxury—placing two metal atoms in the position to bind one molecule of oxygen in the respiratory organs, and to free it again in an oxygen-consuming tissue. The red hemoglobin and a derivative, the green chlorocruorin, achieve this with only 140 amino acids per polypeptide chain and thus attain, with the same effort of protein material, a four-fold oxygen binding capacity. The third known group of respiratory proteins, the pink hemerythrins, with a five-fold oxygen transport capacity, does an even better job in this respect. Therefore, one might suggest that hemocyanin, which in most groups was inefficient to compete with the more economical pigments, is a dead-end product of evolution. However, looking at their distribution among the Invertebrata, we observe that hemocyanin occurs excessively in highly complex animals whereas the more primitive phyla dispose of either hemoglobin, chlorocruorin, or hemerythrin (Prosser, 1973; Bonaventura and Bonaventura, 1980). This is surprising because extracellular hemoglobins as well as red blood cell hemoglobins occur sporadically in molluscs as well as in arthropods (foremost in lower taxa), and thus have been available phylogenetically. What possible advantage made hemocyanin successful over hemoglobin in presumably 100,000 highly advanced animal species, despite its considerably lower oxygen binding capacity? This can be speculated at least for arthropods. As with hemoglobin, the reversible oxygen binding of the blue protein is enhanced, modulated, and adapted by so-called "allosteric" phenomena, based on complex interactions between subunits. Arthropod hemocyanins display this functional plasticity to an extent unknown for hemoglobin or any other allosteric protein (Loewe, 1978; Mangum, 1985). Moreover, no hemoglobin found in a possible candidate for the ancestral arthropod or mollusc exhibits an appreciable allosteric behavior. Among the Invertebrata, the heme proteins with more than token cooperativity are, with a single exception (the bivalve *Scapharca inaequivalvis:* Ascoli *et al.,* 1986), the extracellular ones, which invariably occur in the more specialized groups in a particular taxon. So the ancestors of the hemocyanin-containing molluscs and arthropods were really choosing between a functionally inflexible intracellular hemoglobin (high to moderate oxygen affinity, essentially uncooperative and pH-, inorganic ion-, and organic cofactor-insensitive) and a highly plastic (with respect to oxygen affinity and pH dependence), moderately to highly cooperative, inorganic ion and organic cofactor modulable extracellular hemocyanin. To make the decision easier, it is harder to push a red blood cell-containing hemolymph around in an arthropodan or molluscan circulatory system that lacks capillaries (Mangum, 1985).

Vertebrates evolved a special intracellular hemoglobin which is modulable to some degree, and which compensates for its limited functional plasticity with strong ventilation and circulatory systems which continuously create high gas diffusion rates. Moreover, their interior regulation is efficient enough to embed the hemoglobin in permanent homeostasis, which protects it against drastic changes of the *milieu*. As a result, their blood gained the extremely high oxygen carrying capacity of intracellular hemoglobin. The higher arthropods display sensory and locomotory activities comparable to those of many vertebrates. As in vertebrates, these activities require a continuous, abundant oxygen supply of the involved tissues, despite the more limited vegetative control mechanism in these animals and despite their gills and lungs being lined with chitin that hinders gas diffusion. Their respiratory protein thus plays a much more decisive role as a molecular interface between body tissues and environment. Extremely flexible in function, arthropod hemocyanin compensates for environmental and physiological changes during the life-cycle of the organism (Mangum, 1980; Mangum, 1983), and it also has demonstrated flexibility in the evolution of countless crabs, crayfishes, shrimps, isopods, scorpions and spiders, each adapted to a special aquatic or terrestrial situation.

The "clue" to arthropod hemocyanins is their multigenicity: they are composed of several structurally and functionally different types of subunits. This has been studied intensively worldwide during the last decade (for review: Linzen, 1983). The phylogeny of those subunits, and of the various oligomers, is now well understood. In addition we learned that the native aggregation level, and also most probably the oxygen binding characteristics, are ultimately dependent on the special subunit composition.

MATERIALS AND METHODS

Animal sources, and the specificity of the applied antisera (raised in rabbits) are described elsewhere (Markl *et al.*, 1986a). Other methods are described in detail in the cited literature. However, for a better understanding of the following discussion, a brief description of the major immunochemical approaches may be useful. Immunochemists use very powerful and sensitive techniques (*e.g.*, radioimmunoassay, enzyme-linked immunoassay) which enables the calculation of immunological differences between proteins in 1% steps by successively quantitating the immune reaction between each of the proteins and a suited antiserum. Unfortunately, in our case those methods were inadequate because the hemocyanin subunit samples, especially from crustaceans, were uncontrollably contaminated by self-reassembled aggregates (if the dissociation conditions were too gentle), or by partially denatured subunits (if we chose more drastic conditions). Those contaminants display special immune reactions: compared to subunits, aggregates react at least ten-fold stronger, and denaturation, (*e.g.*, with urea) spirited away intraspecific as well as interspecific immunological differences between hemocyanin subunits (Lamy *et al.*, 1981a; Stöcker, 1984; Kempter *et al.*, 1985). Therefore our data would have been seriously falsified had we used a "blind" quantitative method.

Crossed immunoelectrophoresis

We instead applied the crossed immunoelectrophoresis which, though only being semi-quantitative and less sensitive (soluble immune complexes escape the detection), allows a selective comparison of the desired components in a particular sample. This two-dimensional technique (Weeke, 1973) in the first dimension separates proteins in an agarose gel according to charge differences. In the second dimension the proteins migrate, again electrophoretically, into an antiserum-containing agarose gel. Each protein which reacts with the antiserum forms a curved precipitation line; intersecting lines allow the structural comparison of the respective proteins. In all the patterns discussed here, the anode was on the left in the first dimension. Variations of this technique (tandem-crossed and crossed-line immunoelectrophoresis) enable an intersection of proteins from two different samples.

Immuno blotting

Especially for comparisons of phylogenetically distant subunits, the more sensitive immuno blotting ("Western blotting") technique was used (Towbin *et al.*, 1979; Burnette, 1981). The native subunit patterns obtained by polyacrylamide gel electrophoresis were transferred by diffusion (the currently used electro transfer caused denaturation!) onto an immobilizing nitrocellulose sheet, and then treated with subunit-specific rabbit antibodies. A second goat antiserum against rabbit antibodies was applied subsequently. The second antibodies carry pick-a-back horseradish peroxidase molecules. This enzyme catalyzes a reaction with added diamino benzidine, resulting in a brownish color. Specifically, only those subunit bands which had reacted with the first antibody are stained, whereas other subunits remain invisible. The weak peroxidase activity of hemocyanin (Ghiretti, 1968) was low. This technique is advantageous as soluble immune complexes also are recorded. However, immuno blotting could not serve us exclusively because it was unable to distinguish slight differences between closely related subunits; for this, crossed immunoelectrophoresis was the better tool.

HEMOCYANINS OF THE ARACHNIDA AND OF ALLIED GROUPS

Extent of subunit diversity in tarantula hemocyanin

Ten years ago, when we decided to study the blue blood protein of the North American tarantula, *Eurypelma californicum*, a 4 × 6-mer, few and very contradictory data were available on the number of subunit types present in any one hemocyanin. Polyacrylamide gradient slabgel electrophoresis is an extremely powerful technique used to separate a mixture of very similar proteins. In a buffer system which separates proteins primarily according to charge differences (molecular mass differences played an additional, but minor role), the subunit mixture of tarantula hemocyanin yielded 6 distinct bands: 5 monomers (= single polypeptides) and a dimer. Using a combination of gel chromatography, ion exchange chromatography, and preparative polyacrylamide electrophoresis (today we use immuno affinity chromatography in one single step), each of the six components could be preparatively isolated. In a detergent-containing polyacrylamide gel, which separates polypeptides according to molecular mass differences, we detected that the dimeric subunit is composed of two different monomers (Schneider *et al.*, 1977; Markl *et al.*, 1979a). Thus, the final result was a complex pattern of seven polypeptide chains, which we later have designated as *a* through *g* (Fig. 1). Also a third method visualized the marked diversity of those subunits, namely the crossed immunoelectrophoresis (Fig. 1). No immunological cross-reactivities between any two subunits could be detected (Lamy *et al.*, 1979a). This indicated that the surface structures must be significantly different, which implied major differences in function.

Distribution of the 4 × 6-mer and its derivatives

We found no hemocyanin in the sun spider ("wind scorpion") *Galeodes* sp., and in the watermite *Hydrachna geographica* (Markl *et al.*, 1986a). Both animals possess

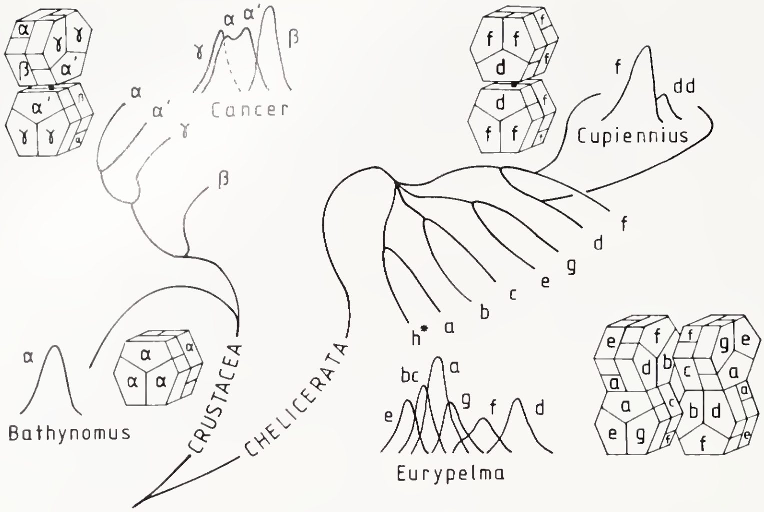

FIGURE 1. Topographical models of the quaternary structure of four arthropodan hemocyanins. The phylogenetic tree shows the relationships between the various subunit types (Kempter *et al.*, 1985; Markl *et al.*, 1986a). The subunits are visualized in their respective patterns of crossed immunoelectrophoresis. Subunit topographies are based on electron microscope analyses of the overall morphology (Van Holde and van Bruggen, 1971; Markl, 1980; Bijlholt *et al.*, 1982; van Bruggen, 1983), and were derived from the combined results of four different approaches: (i) determination of subunit compositions and stoichiometries, (ii) analysis of oligomeric dissociation fragments in comparison with the whole molecule, (iii) reassembly experiments with various subunit combinations, (iv) direct observation in the electron microscope after having decorated the native particles with subunit-specific antibodies (respectively antibody fragments). Beyond the 1 × 6 level, for the formation of the original aggregate the presence of all subunit types is ultimately required. This reflects perfectly the distinct structural roles played by these subunits.

*: Subunit *h* is restricted to scorpion hemocyanin (see Fig. 2).

1 × 6 hemocyanin of the deep sea isopod *Bathynomus giganteus* (a gift of M. Brenowitz) is exclusively composed of alpha subunits (Van Holde and Brenowitz, 1981, and unpub. data).

2 × 6 hemocyanin of the spider *Cupiennius salei* is composed of a disulfide-bridged dimer *d-d* and 10 monomers *f* (Markl, 1980). 2 × 6 hemocyanin of the crab *Cancer pagurus* consists of a non-covalent dimer alpha′-alpha′, a central 4-beta cluster, and, more peripheral, 2 alpha and 4 gamma subunits. A certain flexibility in substituting gamma subunits for alpha appears to exist. 2 × 6 hemocyanin from the freshwater crayfish *Astacus leptodactylus* is constructed correspondingly, but contains a disulfide-bridged dimer. The crab *Callinectes sapidus* and the lobster *Homarus americanus* fit into the scheme, although in those cases no dimer could be identified (Markl *et al.*, 1983; Stöcker *et al.*, 1986).

4 × 6 hemocyanin of the tarantula *Eurypelma californicum* is constructed of a central tetrameric *bc-bc* ring, symmetrically surrounded by 20 monomers: one *a, d, e, f,* and *g* in each basic hexamer. Most probably, peripheral bridges between the two 2 × 6-meric halves are formed via *f-f.* According to Markl *et al.* (1981d). Comparable results stem from scorpion and xiphosuran hemocyanins (Lamy *et al.*, 1981b, 1983b).

a well-developed trachea system and therefore probably have no need of a respiratory pigment. However, as indicated in Figure 2, 4 × 6-mers composed of multiple subunits are present in whip scorpions and whip spiders (Markl *et al.*, 1978, 1979b). A breakthrough like that made with *Eurypelma* hemocyanin was achieved in the analysis of the 4 × 6 hemocyanin of another arachnid, the scorpion *Androctonus australis,* and the 8 × 6 hemocyanin of a xiphosur, the horseshoe crab *Limulus polyphemus* (Hoylaerts *et al.*, 1979; Lamy *et al.*, 1979b, c; Markl *et al.*, 1979b; Brenowitz *et al.*, 1981). *Androctonus* hemocyanin contains an eighth subunit type, designated by us as *h,* and *Limulus* hemocyanin shows two variations of subunit *g,* but for the rest both molecules are composed like *Eurypelma* hemocyanin (Fig. 2). This correspondence was recently confirmed by comparative immunochemistry: each subunit of the tarantula has a

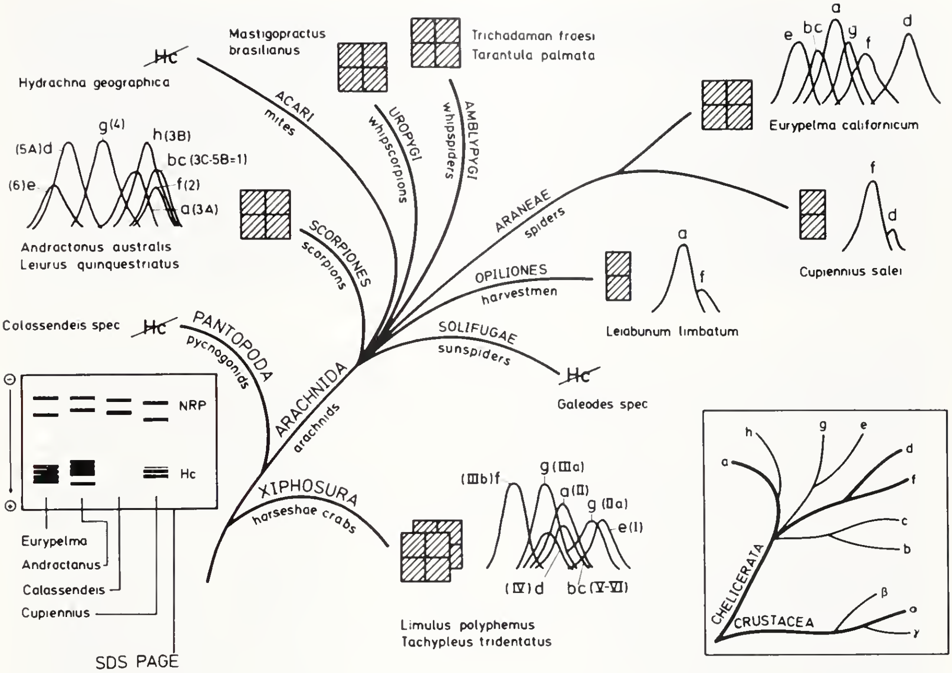
Hc
Hydrachna geographica
Mastigopractus brasilianus
Trichadaman froesi
Tarantula palmata
a
e bc g f d
Eurypelma californicum
ACARI mites
UROPYGI whipscorpions
AMBLYPYGI whipspiders
(5A)d
g(4)
h(3B)
bc(3C-5B=1)
(6)e
f(2)
a(3A)
Andractonus australis
Leiurus quinquestriatus
SCORPIONES scorpions
ARANEAE spiders
f
d
Cupiennius salei
a
f
OPILIONES harvestmen
Leiabunum limbatum
Colossendeis spec
Hc
PANTOPODA pycnogonids
SOLIFUGAE sunspiders
Hc
Galeodes spec
NRP
Hc
ARACHNIDA arachnids
XIPHOSURA horseshoe crabs
(IIIb)f
g(IIIa)
a(II)
g(IIa)
e(I)
(IV)d
bc(V-VI)
Limulus polyphemus
Tachypleus tridentatus
Eurypelma
Andractonus
Colossendeis
Cupiennius
SDS PAGE
h g e
a
d
f
c
b
CHELICERATA
CRUSTACEA
β
α
γ

FIGURE 2. Distribution of hemocyanin among the subphylum Chelicerata. The patterns of crossed immunoelectrophoresis show the respective subunit composition; hatched squares symbolize the native aggregation states (8 × 6, 4 × 6, 2 × 6; small squares correspond to hexamers *). Immunologically homologous subunits are identically labeled using the designations *a–h* established for spider hemocyanins. Scorpion and xiphosuran subunits are additionally labeled according to their original designations (Lamy *et al.*, 1979a, b, c; Markl, 1980). The results from *Tachypleus* and *Leiurus* (Lamy *et al.*, 1979c; Markl *et al.*, 1984) are not discussed in the text, but fit well into the scheme. Whip spider and whip scorpion hemocyanin subunits were studied only by electrophoresis (Markl *et al.*, 1979b). Opilionids possess 2 × 6 hemocyanin (Kempter *et al.*, 1985), whereas no hemocyanin was found in *Galeodes, Hydrachna,* and *Colossendeis* (Markl *et al.*, 1986a). The figure is taken from Markl *et al.* (1986a).

Left insert: polyacrylamide gel electrophoresis (PAGE) of hemolymph proteins in the presence of a detergent (sodium dodecyl sulphate = SDS), showing that the sea spider *Colossendeis* possesses no hemocyanin (Hc), but it does possess the typical arachnidan non-respiratory protein (NRP). This was confirmed by immuno blotting. Comparable unpublished results were recently obtained with the pycnogonid *Nymphon* sp.

Right insert: phylogenetic relationships between the eight cheliceratan subunits according to the combined information of Lamy *et al.*, 1983a, Markl *et al.*, 1984, and Kempter *et al.*, 1985. Broader lines indicate that with anti-crustacean alpha antiserum, subunits *a, d,* and *f* are recognized best.

* For better comparison with the current literature: the sedimentation coefficients of the four aggregates are 60S (8 × 6 = 48-mer), 35S (4 × 6 = 24-mer), 24S (2 × 6 = 12-mer), and 16S (1 × 6 = 6-mer).

homologous subunit in scorpion and horseshoe crab hemocyanin (Lamy *et al.*, 1983a; Markl *et al.*, 1984; Kempter *et al.*, 1985). Moreover, a phylogenetic tree of the subunits could be derived: the pairs *d/f, e/g, b/c,* and *a/h,* respectively, are phylogenetically closely connected (Fig. 1, and right insert in Fig. 2).

Although at that point the various subunits still could be neutral features that have been inherited because they weren't selected against, the heterogeneity appeared as a basic structural design worth conserving in evolution at least since that time when the progenitors of arachnids and xiphosurs diverged from each other. Fossils show that this happened during the Siluran era, more than 400 million years ago (Tiegs and Manton, 1958; Bergström *et al.*, 1980). All the more surprisingly, 4 × 6 hemocyanin was discarded twice in rather advanced arachnidan groups, and replaced by relatively simple 1 × 6 and 2 × 6 hemocyanins (Fig. 2). This occurred in the harvestmen (daddy-long-legs; Kempter *et al.*, 1985) and, independently, in certain spiders (Wibo, 1966; Markl *et al.*, 1976, 1983, 1986a). This phenomenon has been studied intensively, especially in spiders. The blood of the large Central American hunting spider *Cupiennius salei* contains both 1 × 6 and 2 × 6 hemocyanin. The 1 × 6-mers showed electrophoretically 5 different subunit bands. However, to our suprise, these components were completely identical immunologically (Markl and Kempter, 1981a). All five could be correlated immunologically with subunit *f* of *Eurypelma* (Markl *et al.*, 1984; Kempter *et al.*, 1985). 2 × 6-mers, present as a main component of *Cupiennius* blood, are also composed of *f*-homologons, but also contain a dimeric subunit (Fig. 1). This dimer is immunologically related to, but somewhat distinct from the monomers (Markl, 1980) and, as revealed recently by immuno blotting, predominantly related to *Eurypelma* subunit *d* (Markl *et al.*, 1986a). Thus, the 2 × 6 hemocyanin of *Cupiennius* can be derived phylogenetically from a 4 × 6-mer progenitor.

The architecture of Cupiennius *hemocyanin*

Parallel to the comparative studies described so far, we attempted from the beginning to analyze hemocyanin subunits with respect to their reassembly behavior, native stoichiometry, and topologic position in the respective oligo-hexamer. Our goal was to reveal principles of the blue protein's architecture. We used a variety of analytical methods, but especially polyacrylamide gel electrophoresis, immunochemistry, and electron microscopy. Each of the monomeric *f* isozyms of *Cupiennius salei* hemocyanin formed regular homo-hexamers in reassembly experiments; to reorganize the dodecamer, the disulfide-bridged dimer *dd* also was required (Markl, 1980; Markl and Kempter, 1981a). Stoichiometrically, each 2 × 6 particle contained one copy of *dd* and ten copies of *f*. Electron microscopy indicated that a one-point contact between the two hexameric halves could be assumed (Fig. 1). Reducing agents, which cleaved the isolated dimer into single *d* monomers, also cut the 2 × 6-mer down to hexamers. Together, these techniques strongly indicated, but only indirectly, that the dimer was an inter-hexamer bridge—how could this be tested more rigorously? Immuno labeling experiments, as described below, were unsuccessful because of the marked immunological similarity between *Cupiennius dd* and *f*. The solution came unexpectedly: when we monitored the dissociation of a 2 × 6 fraction electrophoretically, we observed that the process passed over a stable oligomeric fragment of unusual size. Molecular mass determinations characterized it as heptamer. In a carefully directed electron microscopic survey we indeed detected a strange, yet undescribed molecule: a regular hexamer with a protruding particle (Fig. 3). We isolated this heptamer and, by reducing agents, cut off the protrusion—obviously a fragment of the molecular bridge. The products of this cleavage were hexamers and single subunits *d*. Thus indeed, the protrusion was identified as the half of the dimer. The resulting model of quaternary structure (Fig. 1) was, among all hemocyanin models, the first which showed a directly localized subunit type in its topologic position (Markl, 1980).

The 4 × 6-mer—a symmetrical mosaic

Computer analysis of electron microscope images revealed the general morphology of *Eurypelma californicum* hemocyanin particles (Bijlholt *et al.*, 1982): the two basic hexamers within a half-structure are rotated against each other in a right angle and come in close contact; the 2 × 6-meric halves keep more distance and are slightly tilted with respect to each other (Fig. 1).

Since two subunit types form a 2 × 6-mer, the necessity of five more different components to make the protein twice as large was difficult to see, and we asked

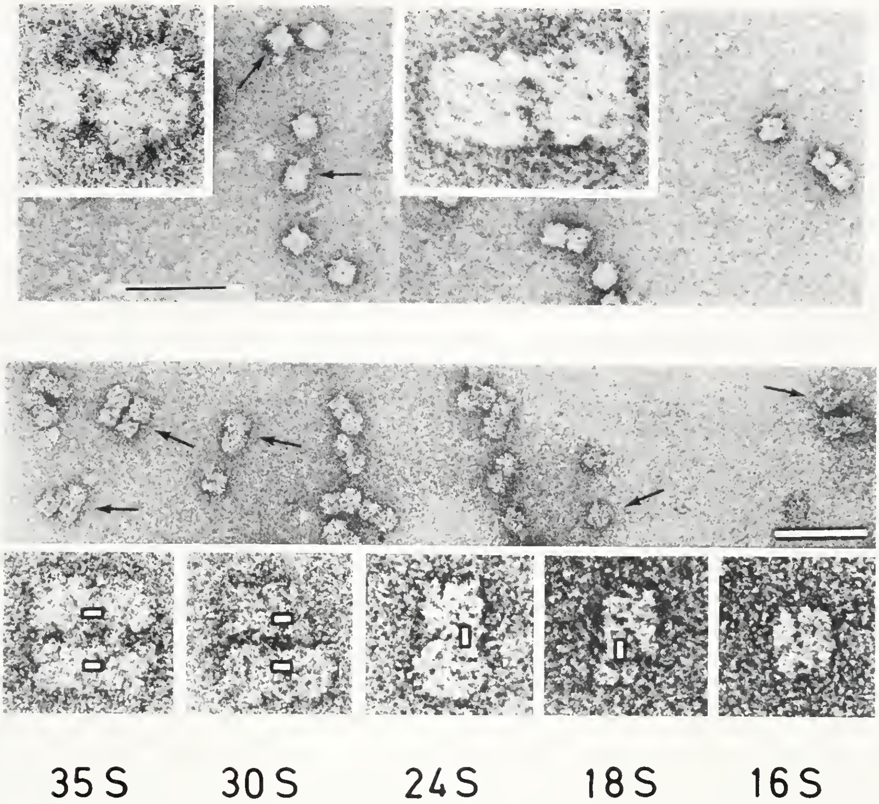

FIGURE 3. *Above:* electron micrographs of hemocyanin molecules from the spider *Cupiennius salei*, negatively stained with 1% unbuffered uranyl acetate. Besides 1 × 6 and 2 × 6 aggregates, heptameric intermediate structures are visible (arrows). The bar represents 50 nm. The left magnified image shows a heptamer with its protruding particle, which could be identified as half-dimer *d*. The right enlargement shows a 2 × 6 molecule in the same orientation. From Markl (1980).

Below: electron micrographs of negatively stained hemocyanin particles from the tarantula *Eurypelma californicum*. The molecules were obtained after 3 hours dialysis of native 4 × 6-mers against pH 9.6, which caused a partial dissociation into oligomeric fragments (upper panel, the bar represents 50 nm). The magnified images (lower panel) have the position and orientation of the heterodimeric subunit *bc* indicated, and are from left to right: 4 × 6-mer (= 24-mer, sediments in the analytical ultracentrifuge with 35S); 3 × 6-mer with one additional subunit protruding in the gap (= 19-mer, 30S); 2 × 6-mer (= dodecamer, 24S); 1 × 6-mer with an additional subunit protruding (= heptamer; 18S); 1 × 6-mer (= hexamer, 16S). The figure is taken from Markl *et al.*, 1981c.

ourselves whether all of these components are really incorporated in the same hemocyanin particle. Using various analytical methods, we tried to detect a heterogeneity of the 4 × 6 molecules of *Eurypelma;* finally we were convinced that indeed only a single type of 4 × 6-mer exists (Markl *et al.*, 1980). The 7 subunits, designated *a* through *g*, are present in constant proportions: $4a + 2b + 2c + 4d + 4e + 4f + 4g = 24$ (Markl *et al.*, 1980, 1981a). The self-assembly process of the 4 × 6-mer is characterized by a striking specificity: reassembled molecules equal native molecules in the stoichiometry of their subunit composition (Decker *et al.*, 1980). None of the subunits was able to self-assemble to homo-hexamers, in contrast to certain horseshoe crab and scorpion hemocyanin components (Lamy *et al.*, 1977; Bijlholt *et al.*, 1979; Brenowitz *et al.*, 1983). A series of reassembly experiments with all possible subunit combinations revealed that indeed each subunit type is required to build up the oligomeric architecture. A substitution by another subunit is not successful: the reassembly process will stop at a certain level, which is typical for each subunit combination (Markl *et al.,* 1981b, 1982). Most of the intermediate structures obtained morphologically corresponded to one of the dissociation fragments described below (Fig. 3). Stable 4 × 6 particles, with a "normal" appearance in the electron microscope are only reorganized in the presence of all seven components. After this, our goal was to localize each subunit within the native 4 × 6 particle. Again, an essential part was the investigation of stable oligomeric dissociation fragments. We attempted a carefully directed search for heptamers, which we indeed found (Fig. 3), together with "conventional" dodecamers and hexamers (Markl *et al.,* 1981c). Additionally, a new fragment appeared: a 19-mer, composed of 3 hexamers and a small protrusion in the gap (Fig. 3). Isolation and analysis of the 4 fragments revealed that the various copies of the 7 subunits are symmetrically distributed among the 4 × 6 particle (Markl *et al.,* 1981a). The exact topology was determined by direct localization of subunits in the electron microscope after having decorated the 4 × 6-mer with subunit-specific antibody fragments (Markl *et al.,* 1981d). A ringlike *bc-bc* tetramer forms the central core which connects the hexameric quarter-structures. Presumably, two *f-f* homodimers achieve more peripheral contacts between the 2 × 6-halves. Within each 2 × 6-mer, subunits *a* and *d* are involved in the inter-hexamer connection, whereas subunits *e* and *g* are arranged peripherally. This model of quaternary structure (Fig. 1) is in agreement with a model which was published in the same year for the 4 × 6 hemocyanin of the scorpion *Androctonus australis* (Lamy *et al.,* 1981b).

8 × 6 hemocyanin of the horseshoe crab *Limulus polyphemus* is dissociable into 4 × 6 half-molecules, which morphologically correspond in detail with native *Androctonus* and *Eurypelma* 4 × 6-mers (Bijlholt *et al.,* 1982), and consist of an immunologically related set of subunits (Lamy *et al.,* 1983a; Kempter *et al.,* 1985). Hybrid hemocyanin molecules ("protein chimaeras"), which we obtained by co-reassembly of subunits from the three species, further underlined this structural relationship (van Bruggen *et al.,* 1980). The recently published subunit topology of this largest of all arthropod hemocyanins indeed confirmed its far-reaching homology with tarantula and scorpion hemocyanin (Lamy *et al.,* 1983b).

Hemocyanin subunit structure, and the evolution of spiders

The striking conservatism of the 7 subunit/4 × 6 structure raised the question, what happened with *Cupiennius* to cause such a dramatic change in its design? To find an answer we attempted a broad survey including 40 species of spiders from 25 families (Markl *et al.,* 1983, 1986a). Previous taxonomic schemes for the higher classification of spiders at the family level have been based on adult morphology or behavior, a topic on which there is currently substantial disagreement (Eberhard, 1982). H. W. Levi stated in 1978: "Spider classification at the present is in chaos." (Personal remark, cited in: R. F. Foelix. 1979. *Biologie der Spinnen.* Thieme, Stuttgart). To use spider hemocyanin as a taxonomic character should be advantageous, because in contrast to most other tried characters the direction of its evolution seems clear: the 2 × 6-mer is a derivative of the 4 × 6-mer and not *vice versa,* because the 4 × 6-mer also occurs in other Chelicerata. According to current textbook taxonomy, the order of spiders (Araneae) contains four suborders: Mesothelae (which were not studied by us), Orthognatha (*e.g., Eurypelma*), Cribellata, and Labidognatha. The Labidognatha are further subdivided in Haplogynae (with simple sex organs) and Entelegynae (with complex sex organs). *Cupiennius* is an entelegyne spider.

The four orthognath families studied all possess 4 × 6-mers with a subunit composition corresponding to that of *Eurypelma* hemocyanin; a homologous structure which also occurs in seven entelegyne families (Fig. 4). Eleven other entelegyne families, however, dispose of hemocyanins which are clearly homologous to that of *Cupiennius* (Fig. 4). Their extreme immunological similarity strongly supports the hypothesis that the last group is a natural, monophyletic taxon. We therefore propose to designate this taxon as "Neo-Entelegynae," and the remaining entelegyne spiders, defined by their 4 × 6 hemocyanin, as "Arch-Entelegynae" (Markl and Runzler, 1986).

It was very interesting for us to detect in a cribellate spider an intermediate step in the transition from the 7-subunit to the 2-subunit particle: *Filistata insidiatrix* possesses only hexameric hemocyanin (This contradicts Wibo, 1966, who reported 4 × 6 hemocyanin for this species). The patterns of crossed immunoelectrophoresis showed five subunit peaks (Fig. 4). Absent was heterodimer *bc*, which makes the presence of only hexamers in this spider understandable: in *Eurypelma californicum* 4 × 6 hemocyanin, *bc* forms a tetrameric core (Fig. 1), which in reassembly experiments was indispensable to exceed the hexamer level (van Bruggen *et al.*, 1980; Markl *et al.*, 1982). *Amaurobius fenestralis*, the second cribellate spider that we investigated, possesses a 2 × 6 hemocyanin with the typical subunit composition of our Neo-Entelegynae (Fig. 4). Alternatively, the spider *Uroctea durandi*, which morphologically appears to be a very close relative of certain cribellate species (Kullmann and Zimmermann, 1976), belongs to our Arch-Entelegynae according to its hemocyanin structure. The Cribellata possess two unique devices: a typical spinning plate, the cribellum and, at their fourth pair of legs, a special comb to brush silk, the calamistrum. Because a repeated, independent evolution of these structures is highly improbable, most experts treat the Cribellata as a natural, monophyletic taxon (*e.g.*, Bristowe, 1938; Levi, 1966; Lehtinen, 1967). However, the striking morphological similarity between various Cribellata and particular entelegyne species stimulated the idea that cribellate characters have been repeatedly and independently lost in spider evolution (Lehtinen, 1967). Our data strongly support this hypothesis, and indicate that at least all the Neo-Entelegynae, and probably the entire labidognath suborder, stem from cribellate progenitors. Compared to the various schemes based on other characters, our results agree substantially with Lehtinen (1967, 1978), despite minor differences which predominantly are refinements.

The haplogyne spider *Dysdera crocata* possesses a hexameric hemocyanin like *Filistata*, but composed of only two different subunits in comparable proportions; immunologically they correspond to *Eurypelma f* and *d* (Fig. 4). The close relationship to *Cupiennius* hemocyanin is obvious; however, the specific structural role of subunit *d* as hexamer linker (Markl, 1980) is not (yet?) established in *Dysdera*. Thus *Dysdera*, and probably the entire group of haplogyne spiders (although their monophyletic nature is doubted) are either late descendents of a neo-entelegyne spider family, or indeed have conserved molecular features of the transition phase between the two hemocyanins. It should be noted that Lehtinen (1967) postulated a close relationship between Filistatidae and Haplogynae, which supports the second assumption. Although presently we are unable to decide with lasting certainty, whether *Filistata* and *Dysdera* hemocyanin represent true "missing links," they can serve as evolutionary models. It may well be that an ancestral cribellate spider, for whatever reason, lost its ability to genetically express the heterodimer, and thus was restricted to hexameric hemocyanin with possibly drastic (though obviously not lethal!) negative consequences: drop of functional plasticity, increase of colloid osmotic pressure, and blood viscosity. In the descendents of this "genetic cripple," three more subunits (probably unnecessary to bring a hexamer to function) disappeared from the phenotype, and later a new mode of inter-hexamer bridging was invented. This was the starting signal for the evolution of a large variety of highly advanced trappers, hunters, and jumpers (Fig. 4). Whether this new hemocyanin, possibly the result of an evolutionary accident, conceals any functional advantage over the 4 × 6-mer remains an interesting, unanswered question.

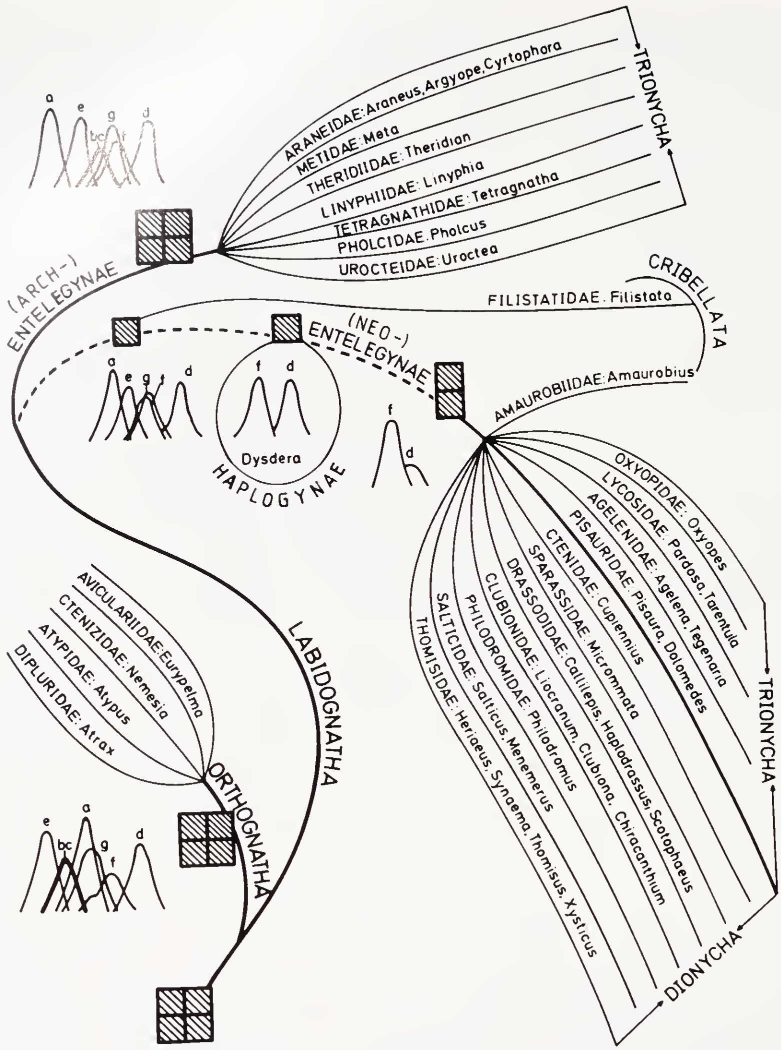
ARANEIDAE: Araneus, Argyope, Cyrtophora
METIDAE: Meta
THERIDIIDAE: Theridion
LINYPHIIDAE: Linyphia
TETRAGNATHIDAE: Tetragnatha
PHOLCIDAE: Pholcus
UROCTEIDAE: Uroctea
TRIONYCHA
(ARCH-) ENTELEGYNAE
CRIBELLATA
FILISTATIDAE: Filistata
(NEO-) ENTELEGYNAE
AMAUROBIIDAE: Amaurobius
Dysdera
HAPLOGYNAE
OXYOPIDAE: Oxyopes
LYCOSIDAE: Pardosa, Tarentula
AGELENIDAE: Agelena, Tegenaria
PISAURIDAE: Pisaura, Dolomedes
CTENIDAE: Cupiennius
SPARASSIDAE: Micrommata
DRASSODIDAE: Callilepis, Haplodrassus, Scotophaeus
CLUBIONIDAE: Liocranum, Clubiona, Chiracanthium
PHILODROMIDAE: Philodromus
SALTICIDAE: Salticus, Menemerus
THOMISIDAE: Herioeus, Synaema, Thomisus, Xysticus
TRIONYCHA
DIONYCHA
AVICULARIIDAE: Eurypelma
CTENIZIDAE: Nemesia
ATYPIDAE: Atypus
DIPLURIDAE: Atrax
LABIDOGNATHA
ORTHOGNATHA
a
e
g
d
bc
f

FIGURE 4. Phylogenetic tree of spiders as deduced from their hemocyanin subunit composition. Typical subunit patterns of crossed immunoelectrophoresis, and symbols for the respective native aggregation state (1 × 6, 2 × 6, 4 × 6) are shown. The 7 subunit/4 × 6-mer occurs throughout the Orthognatha and, immunologically somewhat different though doubtlessly related, in 7 labidognath families, which cover orb-weavers and other stationary trionychan (= three-clawed) species. The 2 subunit/2 × 6-mer is typical for another 11 labidognath families, including—besides some net spiders (Agelenidae)—a large variety of free hunters: lurking seizers, sudden jumpers, and tenacious runners. Due to the extremely close immunological relatedness of the subunits of all 2 × 6-mers, these three-clawed (Trionycha) and two-clawed (Dionycha) spiders most probably represent a monophyletic taxon. The transition of 4 × 6 in 2 × 6 hemocyanin is conserved, or at least modeled, within the cribellate family *Filistatidae* (*Filistata insidiatrix*) and the haplogyne family Dysderidae (*Dysdera crocata*), which both possess 1 × 6 hemocyanin, but with a different subunit composition. Note that *Filistata* hemocyanin lacks subunit *bc* which in *Eurypelma californicum* reassembly experiments was indispensable to exceed the 1 × 6 level (Markl *et al.*, 1982). A second cribellate spider, *Amaurobius fenestralis*, possesses 2 × 6 hemocyanin.

According to our data, Labidognatha and Cribellata are not separable, but represent phylogenetically intersecting, nested groups. Entelegynae is a polyphyletic taxon, containing two natural groups, which are disconnected by a haplogyne/cribellate cluster. We have therefore proposed to substitute "Entelegynae" for two new monophyletic terms, namely "Arch-Entelegynae" and "Neo-Entelegynae" (Markl and Runzler,

The sea spiders—a phylogenetic mystery?

Besides the aquatic xiphosurans and the terrestrial arachnids, in current taxonomy the subphylum Chelicerata includes as a third class the marine sea spiders (Pycnogonida = Pantopoda), although the phylogenetic position of these indeed spider-like, often incredibly tiny animals is uncertain. Among experts it is still debated whether they are arachnids, non-arachnidan chelicerates, or a completely independent arthropod subphylum (Bergström *et al.*, 1980). Also remarkably, they have no respiratory organs—very unusual among arthropods, especially aquatic species, in which cutaneous gas exchange is blocked by an impermeable exoskeleton. Hoping to detect a phylogenetically interesting new version of the blue protein, we have studied the minute *Nymphon* sp. from the North Sea and the huge Antarctic *Colossendeis* sp. (Markl *et al.*, 1986a; and unpub. data). Unfortunately, polyacrylamide electrophoresis of their blood yielded no trace of hemocyanin-like protein chains.

Earlier, Redmond and Swanson (1968) reported that spectral-absorption curves of the blood of another giant Antarctic sea spider, *Ammothea striata,* showed no evidence of a blood respiratory pigment. *Nymphon* with its size below 1 cm, and body and extremities as thin as threads, is probably qualified for cutaneous gas exchange. However, the absence of an oxygen carrier in *Colossendeis* and *Ammothea,* which both exceed in size a large tropical spider, but live in the oxygen-poor aquatic environment, is surprising. On the other hand, many cold-blooded animals found in polar seas lack respiratory pigments because of the high oxygen solubility in icy-cold body fluids.

Instead of hemocyanin as desired, in both species we observed electrophoretically two prominent polypeptide chains with molecular masses around 110,000—the typical range of the second major blood protein of spiders and other arachnids (left insert in Fig. 2). This protein was described by us in earlier studies and designated as a "non-respiratory protein" (Markl *et al.*, 1976, 1979b; Linzen *et al.*, 1977). In case of the tarantula *Eurypelma californicum,* it was recently further characterized as lipoprotein associated with a carbonic anhydrase (Stratakis and Linzen, 1984). By immuno blotting, indeed a structural relationship between the non-respiratory protein of the tarantula, and the pycnogonid protein was shown (Markl *et al.*, 1986a). According to the available data this protein is restricted to scorpions, whip scorpions, whip spiders, and spiders, and was detected in neither xiphosuran nor crustacean blood; non-respiratory blood proteins of those two groups are composed of considerably smaller polypeptide chains (Markl *et al.*, 1979b, c). Thus, the occurrence of this protein is a good argument in favor of a close phylogenetic relationship between the sea spiders and the arachnids.

SUBUNIT DIVERSITY IN CRUSTACEAN HEMOCYANINS

At least 600 million years ago, the branch leading to the Chelicerata was separated from the line leading to the Crustacea (Tiegs and Manton, 1958; Schram, 1982). Since today both groups have hemocyanins based on a hexameric architecture, this hemo-

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1986). Our results agree in principal with the evolution scheme of Lehtinen (1967), which is based on morphological characters.

The figure was slightly modified from Markl *et al.* (1986a). The following species were employed: *Agelena labyrinthica, Amaurobius fenestralis, Araneus diadematus, Araneus umbriaticus, Argiope aurantia, Argiope bruennichi, Atrax formidabilis, Atypus affinis, Callilepis nocturna, Chiracanthium elegans, Clubiona terrestris, Cupiennius salei, Cyrtophora citricola, Dolomedes fimbriatus, Dysdera crocata, Eurypelma californicum, Filistata insidiatrix, Haplodrassus signifer, Heriaeus hirtus, Linyphia marginata, Liocranum rupicola, Menemerus taeniatus, Meta segmentata, Micrommata rosea, Nemesia sp., Oxyopes lineatus, Pardosa amentata, Philodromus collinus, Pholcus phalangioides, Pisaura mirabilis, Salticus scenius, Scotophaeus quadripunctatus, Synaema globosum, Tarentula fabrilis, Tegenaria atrica, Tetragnatha extensa, Theridion varians, Thomisus onustus, Uroctea durandi, Xysticus bifasciatus.*

cyanin undoubtedly belonged to their common ancestor. Thus it was of considerable interest to study the subunit composition of crustacean hemocyanins, which are commonly 1 × 6 or 2 × 6 aggregates (the only known exception are the thalassinid shrimps, which possess a tetrahedrical 4 × 6-mer: Miller *et al.*, 1977; van Bruggen, 1983). We applied high resolution electrophoresis techniques, and revealed complex patterns of subunit heterogeneity in various crustacean hemocyanins: between three and eight distinct polypeptides were detected throughout (Markl *et al.*, 1978, 1979c). It was impossible, however, to recognize a common scheme except for the fact that in 2 × 6-mers the heterogeneity was somewhat more excessive than in native single hexamers.

To monitor evolutionary trends, we needed a tool to sort all these subunits into groups of similar function. Because protein function is a matter of surface structure, the tool employed was immunochemistry: each antibody is specifically directed against a fragment of the protein surface, and refuses binding if this epitope is altered. The outcome of a comparative immunochemical analysis of 41 crustacean hemocyanins is described below.

Subunit composition of 2 × 6 hemocyanins from brachyuran crabs

The typical hemocyanin of a brachyuran crab, like the blue crab *Callinectes sapidus*, or the green shore crab *Carcinus maenas*, is a 2 × 6-mer, composed of two immunologically discernible subunit fractions (Rochu and Fine, 1980; Markl and Kempter, 1981a, b; Ghidalia *et al.*, 1985). We have designated them as alpha and beta (Fig. 5). Beta is immunologically unrelated to alpha, which indicates substantial differences in function. Alpha subunits from different crab species are immunologically similar; they remained relatively unchanged after the worldwide radiation of the Brachyura, and therefore were classified by us as "phylogenetically conservative." In a striking contrast, beta subunits differ greatly immunologically among the species, and therefore were classified as "phylogenetically variable" (Markl and Kempter, 1981a). Obviously, during crab evolution, these two subunit types endured very different selection pressures, which again indicates fundamental differences in function.

All in all, we studied 32 species covering a broad environmental and activity range, from shallow water, intertidal, fresh water, and land, and from most agile to rather clumsy (Markl and Kempter, 1981b; Markl *et al.*, 1986a). The representatives of 10 out of 11 crab families possessed alpha and beta subunits (Fig. 5), regardless of how diverse their subunit patterns were electrophoretically. Many Brachyura also have a third hemocyanin subunit, designated as gamma. Gamma appears to be a late offspring of alpha, because their immunological reactions are similar.

The only exception from this general scheme was detected in the family Ocypodidae: the hemocyanins of a fiddler crab (*Uca urvillei*) and of three species of ghost crabs (Genus *Ocypode*) are exclusively composed of alpha subunits (Fig. 5). It was very interesting to detect that *Uca* hemocyanin particles are only single hexamers, which indicates that the absence of beta may be correlated with a restriction to the hexameric level. However, much to our surprise, all three *Ocypode* species possess mainly 2 × 6-meric hemocyanin (Stöcker, 1984; Markl *et al.*, 1986a; B. Johnson, pers. comm.). In contrast to *Uca*, *Ocypode* hemocyanin contains two electrophoretically distinct alpha isozymes (Fig. 5). The cathodic of these immunologically identical proteins migrates in the range of beta, but carries no beta-typical antigen determinants. One could assume that the situation within the Ocypodidae mirrors an ancient trend, leading from single alpha hexamers via alpha/alpha 2 × 6-mers to alpha/beta 2 × 6-mers. However since among the Brachyura, *Uca* and *Ocypode* belong to a rather specialized family, other possibilities had to be considered as well. To clarify this point we had to answer the question of whether beta is an invention of the comparatively modern crabs.

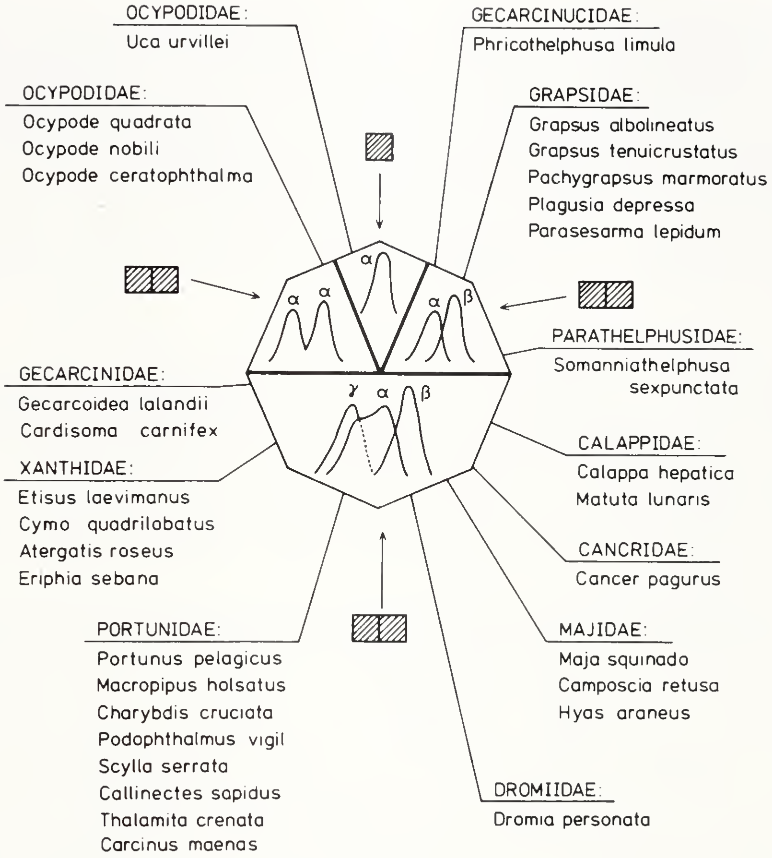
OCYPODIDAE:
Uca urvillei
OCYPODIDAE:
Ocypode quadrata
Ocypode nobili
Ocypode ceratophthalma
GECARCINUCIDAE:
Phricothelphusa limula
GRAPSIDAE:
Grapsus albolineatus
Grapsus tenuicrustatus
Pachygrapsus marmoratus
Plagusia depressa
Parasesarma lepidum
α
α α
α β
γ α β
PARATHELPHUSIDAE:
Somanniathelphusa sexpunctata
GECARCINIDAE:
Gecarcoidea lalandii
Cardisoma carnifex
XANTHIDAE:
Etisus laevimanus
Cymo quadrilobatus
Atergatis roseus
Eriphia sebana
CALAPPIDAE:
Calappa hepatica
Matuta lunaris
CANCRIDAE:
Cancer pagurus
PORTUNIDAE:
Portunus pelagicus
Macropipus holsatus
Charybdis cruciata
Podophthalmus vigil
Scylla serrata
Callinectes sapidus
Thalamita crenata
Carcinus maenas
MAJIDAE:
Maja squinado
Camposcia retusa
Hyas araneus
DROMIIDAE:
Dromia personata

FIGURE 5. Brachyuran crabs arranged according to their hemocyanin subunit composition as analyzed by crossed immunoelectrophoresis. The native aggregation states (1 × 6, 2 × 6) are symbolized. Immunologically corresponding subunits are identically designated. It should be noted that immunologically homogeneous subunit peaks may contain several electrophoretically separated isozymes. Thus, the electrophoretic heterogeneity is generally higher than indicated here (for review: Linzen, 1983).

The tropical species were collected on the Malayan Peninsula. The animals cover a broad environmental range from fairly constant to permanently varying marine *milieus*, from icy to tropic temperatures, and through all stages of land-life: dependent on moist burrows during lowtide (*Etisus*), sun-exposed on rocks (*Plagusia*), in cold freshwater falls (*Phricothelphusa*), in warm stuffy rice fields (*Somanniathelphusa*), in mangrove mud (*Scylla*), in soil burrows (*Cardisoma*), in palmtree forests (*Gecarcoidea*), on hot sandy beaches (*Ocypode*). The smallest (*Cymo*) had a carapace width of less than 2 cm, the largest (*Scylla*) of more than 20 cm. Their activities range from clumsy dwelling (*Dromia*), slow rambling (*Hyas*), hectic rowing (*Matuta*), tenacious waving (*Uca*), fast swimming (*Charybdis*), swift walking (*Carcinus*), and sudden jumping (*Grapsus*), to extremely speedy sand running (*Ocypode*). The figure is taken from Markl *et al.* (1986a).

Spiny lobster 1 × 6 hemocyanin—a possible predecessor?

According to convincing palaeontological records the brachyuran crabs, the latest appearing decapod group, evolved from ancestors related to spiny lobsters (Schram, 1982). Interestingly, spiny lobsters possess 1 × 6 hemocyanin. We investigated the European species *Palinurus vulgaris*, and its American relative *Panulirus interruptus* (Markl *et al.*, 1979c, 1983, 1986a; Stöcker, 1984). Their hemocyanins are composed of an immunologically homogeneous subunit fraction, which corresponds to brachyuran alpha (Fig. 6). *Panulirus* hemocyanin contains a second, cathodic subunit in addition (designated as *c* by Neuteboom *et al.,* 1986) which, to a certain degree, is immunologically related to alpha. Beta-typical antigen determinants were not detected. This is consistent with our definition of the behavior of a gamma subunit, and the component was designated accordingly, although we have no further evidence that *Panulirus* and brachyuran gamma components are really homologous. From its subunit composition, the hemocyanin of a scyllarid (*Scyllarus arctus*), a close relative of spiny lobsters, fits well into the scheme (Fig. 6); however, surprisingly it forms 2 × 6 particles (unpub.). Although the phylogeny of subunit beta, and also the structural requirements for a 2 × 6 formation remained unclear, the situation found within the Palinura at least further supported the hypothesis that alpha subunits were the basic hexamer formers.

Hemocyanins from lobsters, freshwater crayfishes, and shrimps

Geological strata document that, when the first Brachyura appeared 200 million years ago (their great radiation started 140 million years later upon a worldwide formation of shallow water seas), the Astacura were already established (Schram, 1982). Astacura also possess 2 × 6 hemocyanin. In the case of the European freshwater crayfish *Astacus leptodactylus* it is composed of four distinct subunits: a disulfide bridged dimer and three monomers (Markl *et al.,* 1979c; Pilz *et al.,* 1980; Markl and Kempter, 1981b).

Without major difficulties, the dimer and two of the three monomers could be assigned to the alpha/gamma cluster of the Brachyura (Fig. 6). Again, the conservative nature of alpha-typical antigen determinants was confirmed (Stöcker, 1984; Markl *et al.,* 1986a). What remained was the question of a possible relationship between the third *Astacus* monomer and brachyuran beta. By crossed immunoelectrophoresis, anti-*Astacus* antiserum did not precipitate *Cancer pagurus* beta, and *vice versa.* Therefore, for a comparison of structurally distant antigens we applied the immuno blotting technique, which is more efficient. This sensitive method revealed that anti-*Astacus* antibodies specific for the third monomeric subunit preferentially bind to brachyuran beta and *vice versa,* whereas alpha components are only weakly recognized (Markl *et al.,* 1986a). Comparable data stem from the 2 × 6 hemocyanin of the lobster *Homarus americanus,* although in this case the alpha fraction was immunologically homogeneous and no dimer was present; therefore we could not define a subunit alpha′ in this animal (Fig. 6). Hemocyanin from another decapod, the caridean shrimp *Palaemon elegans,* contains only alpha and gamma subunits (Stöcker, 1984; Markl *et al.,* 1986a); however, its native aggregation level is 1 × 6 (Fig. 6)! These results indicated: first, beta is not an invention of the Brachyura, but phylogenetically considerably older, and second, the presence of beta is correlated with the appearance of 2 × 6 particles. The second conclusion is implicated by the exceptions *Ocypode* and *Scyllarus,* which will be discussed below.

It should be noted that in shrimps and crayfishes, gamma subunits are only defined according to their close relationship with alpha subunits; in contrast to alpha and beta, the homology of astacuran or caridean gamma with the respective palinuran and brachyuran components is uncertain. Gamma could have evolved independently from alpha several times.

The origin of subunit beta

It was found recently that 2 × 6 hemocyanin also occurs in a fourth decapodan group, namely the Anomura (Stöcker, 1984; Markl *et al.,* 1986a). Surprisingly, despite a marked electrophoretic heterogeneity, the subunits of 2 × 6 hemocyanins from a galatheid shrimp (*Galathea squamifera*), a hermit crab (*Pagurus bernhardus*), and the coconut crab (*Birgus latro*) were immunologically completely homogeneous (Fig. 6).

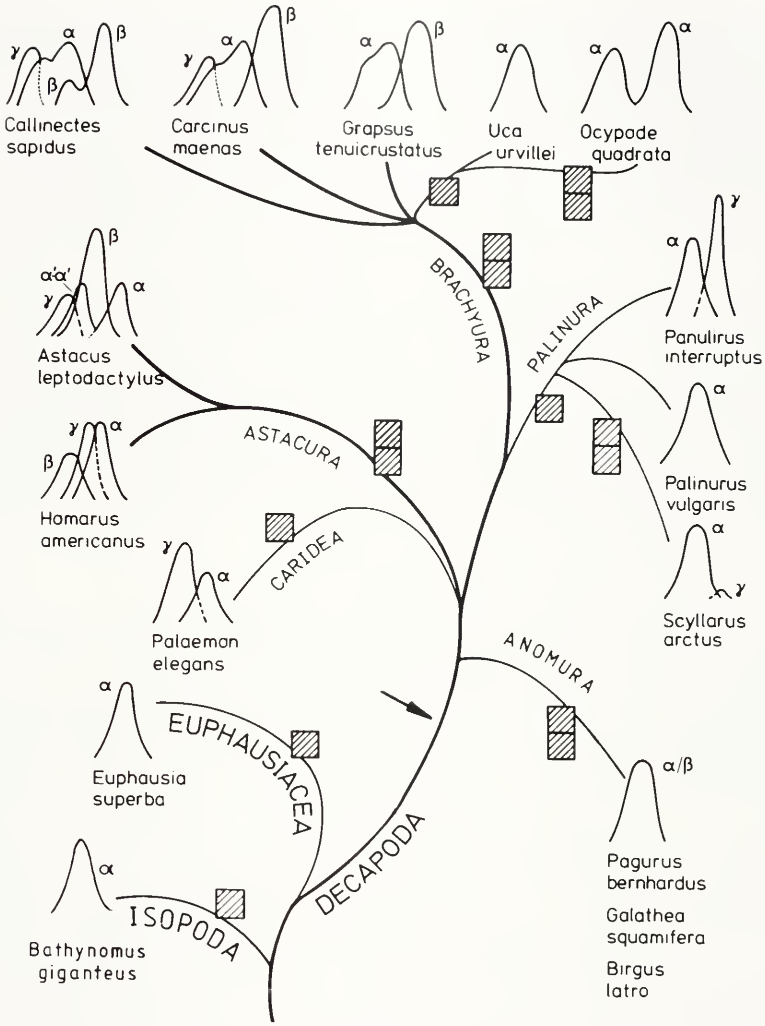
Callinectes sapidus
Carcinus maenas
Grapsus tenuicrustatus
Uca urvillei
Ocypode quadrata
BRACHYURA
Astacus leptodactylus
Homarus americanus
ASTACURA
PALINURA
Panulirus interruptus
Palinurus vulgaris
Scyllarus arctus
Palaemon elegans
CARIDEA
ANOMURA
Euphausia superba
EUPHAUSIACEA
Pagurus bernhardus
Galathea squamifera
Birgus latro
Bathynomus giganteus
ISOPODA
DECAPODA
α
β
γ
α'
α/β

FIGURE 6. The distribution of immunologically corresponding hemocyanin subunits among the Crustacea. The various subunit patterns of crossed immunoelectrophoresis are shown, and the native aggregation states (1 × 6, 2 × 6) are indicated.

It appears that decapodan hemocyanins evolved from an ancient alpha hexamer; the first step was the invention of beta-typical antigen determinants (arrow), which is correlated with the occurrence of 2 × 6 aggregates. Beta was repeatedly lost in later appearing groups (Caridea, Palinura, *Uca/Ocypode*). All alpha subunits are homologous proteins as are all beta subunits. Gamma differs, however: except for their partial identity with alpha, we were unable to identify typical immune reactions which interpose between more distant gamma subunits. Thus, rather than being homologous, they could represent convergent offsprings of different alpha progenitors.

It should be noted that our results, although useful in explaining the phylogenetic coherence between the various subunits, in most cases are insufficient to refine the current phylogenetic tree derived from morphological characters and palaeontological records (Schram, 1982), which is the basis for this scheme. As an exception, our data strongly support an early branch of the Anomura as indicated, which was not derived from classical approaches. The figure was modified from Markl *et al.* (1986a).

Thus, in terms of immunochemistry, only one single subunit type is present. We obtained clear immunological cross-reactions with brachyuran and astacuran alpha but also, and this was the second surprise, with astacuran (not with brachyuran!) beta. The common presence of alpha- and beta-typical antigen determinants on a single subunit cannot be explained as the result of a gene fusion, because the size of the anomuran polypeptide chains is quite within the expected range (Mr = 76,000–83,000). Provided that hemocyanin genes are arranged in a single cluster, one could expect a current rearrangement by unequal crossing-over, which may well explain the anomuran result. However, the marked divergence between alpha and beta in crabs and crayfishes strongly indicates the existence of two different gene clusters like, for example, the case with the mammalian globin genes. It is therefore much more likely to assume that in the Anomura the blue protein has preserved an ancient feature, and that in later appearing species, alpha and beta antigen determinants have been separated by the independent evolution of isozymes. This strongly suggests that the evolution of the decapod 2 × 6-mer indeed began with the appearance of subunit beta, although later beta was repeatedly lost (Palinura, Caridea, Ocypodidae). This, however, was not necessarily tantamount to a permanent restriction to the hexameric level, because alternative, beta-free modes of 2 × 6-mer formation evolved in specialized groups (*Ocypode, Scyllarus*).

Krill and isopod 1 × 6 hemocyanin: models of an ancient design

We tried to demonstrate this by examining the situation outside of the Decapoda. The widespread hemocyanin aggregate of the other Malacostraca is the 1 × 6-mer, which we studied from the Antarctic krill *Euphausia superba* and from the giant deep sea isopod *Bathynomus giganteus* (Fig. 6). The subunits of both proteins exhibited a completely homogeneous peak in crossed immunoelectrophoresis, and corresponded immunologically to subunit alpha of decapodan hemocyanins. Thus, those 1 × 6-mers are entirely composed of alpha subunits (Fig. 1; the different information of Markl *et al.,* 1983, was due to denaturation). It appears, moreover, that *Euphausia* hemocyanin has maintained a considerably ancient surface structure: by immunoelectrophoresis, it was the only crustacean hemocyanin which could be precipitated (in its oligomeric form—not as subunits: see below) by an antiserum raised against tarantula hemocyanin (Van Holde and Brenowitz, 1981; M. Brenowitz, pers. comm.; Stöcker, 1984; Markl *et al.,* 1986a, and unpub. data). This definitely showed that, in arthropods, alpha-typical antigen determinants form the ancestral design of the blue protein's surface.

Mirrors larval crab hemocyanin the blue protein's evolution?

The subunit composition of crab hemocyanins have endured ontogenetic changes (Terwilliger and Terwilliger, 1982). We therefore studied various larval stages of the spider crab *Hyas araneus* and the shore crab *Carcinus maenas.* Hemocyanin from the planktonic zoea, and from the benthonic megalops of both species was composed of a single subunit type, which clearly cross-reacted with adult alpha subunits (Markl *et al.,* 1986a), although electrophoretically it behaved somewhat differently. The native aggregation level of those larval hemocyanins is mainly hexameric, but also 2 × 6-mers have been observed in the electron microscope (unpub.). Thus, the loss of subunit beta in the evolution of some decapoda, and an alternative mode of 2 × 6-mer formation (*Ocypode, Scyllarus*), could have occurred to preserve larval characters.

Recently, we have further monitored the following events in *Carcinus maenas:* after metamorphosis, during the following molting cycles subunit beta appears, although its proportion in the first juvenile crab is still rather low. 2 × 6 hemocyanin particles can be seen in the electron microscope, but the main aggregation level is still hexameric. The second juvenile crab clearly contains mostly 2 × 6 hemocyanin with a considerable proportion of subunit beta. Subunit gamma appears several stages later when the carapace measures 12 mm in width. The typical, stoichiometrically correct subunit pattern of the adults is established when the carapace width measures 22 mm or more (L. E. Precht, B. Steiff, and J. Markl, unpub. data). It is interesting that not only phylogenetically, but also ontogenically, alpha subunits appear first. Also our above presumption that gamma may be a rather late evolutionary product is mirrored in ontogenesis.

A model of crustacean 2 × 6 hemocyanins

The results described above indicate that particular subunits play distinct roles in the formation of 2 × 6 structures. This was closely investigated in two astacuran and two brachyuran species. The overall morphology of these particles, especially the one-point contact between the two hexamers as shown in Figure 1, is described by van Bruggen (1983). Reassembly experiments with isolated subunits alpha, beta, or gamma of the 2 × 6 hemocyanins from the crayfishes *Astacus leptodactylus* and *Homarus americanus,* and from the crabs *Cancer pagurus* and *Callinectes sapidus* showed that in most of the cases, homo-hexamers can be formed (Markl and Kempter, 1981a; Stöcker *et al.,* 1986). However, all three immunologically defined classes of subunits are required to reach the 2 × 6 level (Stöcker *et al.,* 1986). Immunologically identical isozymes are in some cases capable of substituting for each other, especially in *Homarus* and *Callinectes.* Heptameric segments of native 2 × 6-mers were observed in *Astacus,* whereas the 2 × 6 particles of crabs and *Homarus* dissociate via hexamers (Markl *et al.,* 1981c). The inter-hexamer bridge was identified in the case of *Astacus* and *Cancer.* Surprisingly the bridge is not formed by subunit beta as expected. In *Cancer* a particular alpha isozyme (designated as alpha′) has a tendency to dimerize, and connects the two hexamers (Markl *et al.,* 1983). Correspondingly, in *Astacus* the disulfide-bridged dimer is a correlate of alpha. This makes the existence of beta-free 2 × 6 structures as present in *Ocypode, Scyllarus,* and in larval crabs, at least structurally understandable. It should also be noted that 2 × 6 hemocyanins were reported for two species outside of the Decapoda: the isopod *Ligia pallasii,* and the stomatopod *Squilla mantis* (Terwilliger, 1982; van Bruggen, 1983). From our data described above, we would expect a beta-free mode of 2 × 6 formation also in those hemocyanins.

Our next step was to analyze the respective subunit stoichiometries. Finally, labeling of the dodecamers with subunit-specific antibody fragments, or with intact antibody molecules, and observation of the resulting complexes in the electron microscope led to a uniform topological model (Fig. 1). It shows that one dimer alpha′-alpha′ and four copies of beta form a central cluster. Although beta may not be involved directly in the bridge, these four subunits are clearly in the topographical position to play a functional key-role. One alpha occupies the extreme outer edge of each hexamer, and four copies of gamma fill the periphery (Stöcker *et al.,* 1986). Though a preliminary topological model of a crustacean 2 × 6 hemocyanin was already published by Jeffrey (1979), this is the first detailed conception of its architecture.

The connection to the cheliceratan subphylum

We were particularly interested in detecting immunological relationships between crustacean hemocyanin subunits and those from the Chelicerata. By immunoelectrophoresis, a precipitation of crustacean hemocyanins with an anti-chelicerate antiserum, or *vice versa,* was only successful if the hemocyanin was either present in its oligomeric form (see above: *Euphausia*), or denatured in 8 *M* urea (Stöcker, 1984). Therefore, we performed immuno blotting experiments with electrophoretic patterns of native hemocyanin subunits from the horseshoe crab *Limulus,* the scorpion *Androctonus,* the tarantula *Eurypelma,* and the hunting spider *Cupiennius* against anti-alpha antisera from crustaceans. In each case, the total set of chelicerate subunits was recognized (Markl *et al.,* 1986a). Anti-beta and anti-gamma antisera give similar, but much weaker reactions. As judged semiquantitatively, chelicerate subunits related to *Eurypelma a, d,* and *f* are recognized best. This is illustrated in the right insert of Figure 2. It should be interesting to analyze comparatively the 6 × 6 hemocyanin of the myriapod *Scutigera coleoptrata* described by Mangum *et al.* (1985). Our results do *not* mean that there is one particular chelicerate subunit which corresponds to alpha, and another subunit which corresponds to beta; those specializations are certainly late developments. We showed that despite their impressive diversity in recent species, the hemocyanin subunits of all arthropods have maintained—to a different extent—some common ancient surface structures. These features were inherited, unchanged, over at least 600 million years!

INTERACTION OF SUBUNITS IN THE OXYGEN BINDING PROCESS

Recently, several complete amino acid sequences of chelicerate and crustacean hemocyanin subunits were published, the structure of the active copper-site was further elucidated, the conformation of a crustacean 1 × 6-mer was studied in a 3.2 Angström resolution, and the topologic models of *Limulus* and *Androctonus* hemocyanin were considerably refined (Solomon, 1981; Sizaret *et al.,* 1982; Schartau *et al.,* 1983; Schneider *et al.,* 1983; Eyerle and Schartau, 1985; Gaykema *et al.,* 1985; Lamy *et al.,* 1985; Linzen *et al.,* 1985). This information, in the context of the results described here, has demystified, to a considerable degree, the architecture and the evolution of arthropod hemocyanins. Moreover, a wealth of data exists on their function as oxygen carriers (*e.g.,* Van Holde and van Bruggen, 1971; Bonaventura and Bonaventura, 1980; Mangum, 1980, 1983, 1985; Van Holde and Miller, 1982; Antonini *et al.,* 1983; Bridges *et al.,* 1983; Ellerton *et al.,* 1983). However, the processes which determine the typical oxygen binding, and the specific contribution of each subunit type, are still obscure. Presently, *Eurypelma californicum* hemocyanin is the best understood example.

The 4 × 6-mer in action, and the abilities of isolated subunits

Hemocyanin from the tarantula *Eurypelma californicum* is characterized by a relatively low oxygen affinity, a strong normal Bohr effect (= pH sensitivity of oxygen affinity), and an extreme cooperativity (sigmoidity of the oxygen binding curve); additionally, the cooperativity depends considerably on pH (Fig. 7). Upon direct measurements of blood pH and blood P_{O_2} in different tissues during exercise and rest, these properties can be interpreted as highly adaptive with respect to the animal's environment, behavior, and physiology. (Linzen *et al.,* 1977; Loewe, 1978; Angersbach, 1978; Decker *et al.,* 1983a, b; Fincke *et al.,* 1986; Paul, 1986). In contrast, as illustrated in Figure 7, isolated subunits are non-cooperative (hyperbolic binding curve), show a high oxygen affinity, and indicate a complete absence of any Bohr effect. All seven subunits, studied individually, behaved rather uniformly in those aspects (Decker *et al.,* 1979; Markl *et al.,* 1981b, e). Thus, the 4 × 6-mer acts as a system; all its vital abilities are qualitatively new and unexpected, and cannot be predicted from the properties of the subunits. The systemic characters are created by an interaction of the 24 constituents. With subunits in the blood instead of 4 × 6 particles, the animals could impossibly survive. An interesting question was now, at what structural level the respective function occurs?

Events within and beyond the native 1 × 6 fragment

Tarantula hemocyanin again was an exceptionally useful molecule, because the four oligomeric dissociation fragments described above (Fig. 3) could be stabilized in their respective structure over a broad pH range—a fundamental requirement for a

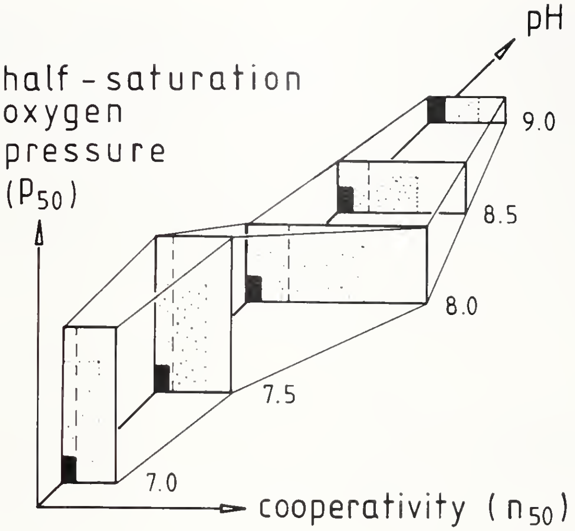

FIGURE 7. Illustration of the functional plasticity of native 4 × 6-meric hemocyanin of the tarantula *Eurypelma californicum*, compared to the functional inflexibility, and the very different behavior, of the isolated subunits.

Subunits (*small black areas*) show a low p50 around 5 mm Hg, a high oxygen affinity. This behavior is independent of pH (= no Bohr effect). Moreover, they are non-cooperative (hyperbolic oxygen binding curve: n50 = 1). All subunits behave similarly (Decker *et al.*, 1979; Markl *et al.*, 1981e). In contrast, the 4 × 6-mer (*large white rectangles*) shows p50 values up to 30 mm Hg (at pH 7.5), or low oxygen affinities. Moreover, it exhibits impressive cooperativities (sigmoid oxygen binding curves); the maximum at pH 8.0 goes up to n50 = 8 and more. Oxygen affinity, and also cooperativity, are *both* strongly pH dependent (Loewe, 1978), which is highly adaptive with respect to the animal's physiology and behavior (Angersbach, 1978). We recently detected that, although the natural resting blood pH of the tarantula is 7.5 (Angersbach, 1978), the *in vivo* behavior of the 4 × 6-mer should be like that illustrated here for pH 8.0; calcium and magnesium ions in native concentrations (4 m*M* each) modulate the function correspondingly (B. Markl and J. Markl, unpub.). All in all, this aggregate of 24 functionally limited components displays completely novel physiological properties. These systemic characters are created by subunit interaction phenomena, and cannot be predicted from the behavior of the isolated subunits.

To study the molecular organization of those interactions, one attempt is to analyze the abilities of oligomeric dissociation fragments (Savel *et al.*, 1983, 1986): for example in the 1 × 6 quarter-structure (areas on the left of the *dashed lines*), the 4 × 6-mer's oxygen affinity is already fully established, but cooperativity reaches only ¼ of the final values.

Another attempt is to analyze reassembled 4 × 6-mers which have a chemically modified subunit incorporated. Modification was done, for example, with mercury(II) ions. A treatment of the whole molecule blocks the interaction processes entirely: the morphologically intact 4 × 6-mer functions like isolated subunits (Markl *et al.*, 1986b). Hemocyanins composed of one mercury-labeled and six unmodified subunit types show a reduced functional plasticity (*spotted areas*), which is believed to be due to the encoupling of the modified subunit from the interaction processes (Markl *et al.*, 1986b).

detailed comparison of their function. Another chelicerate, *Limulus polyphemus*, unfortunately could not fulfill this demand (Brenowitz *et al.*, 1984). In contrast, 1 × 6 hemocyanin fragments of certain crustaceans, like the lobster *Homarus americanus*, the thalassinid shrimp *Callianassa californiensis*, and in the mangrove crab *Scylla serrata*, are stable (Tai and Kegeles, 1971; Arisaka and Van Holde, 1979; Herskovits *et al.*, 1983; Decker *et al.*, 1986a).

An oxygen binding analysis of the isolated quarter-structure (1 × 6-mer) of *Eurypelma* hemocyanin revealed that already on this level, the typical oxygen affinity and the full Bohr effect of the 4 × 6-mer are established; however, cooperativity reaches only 25% of the native value (Fig. 7; Savel *et al.*, 1983). The heptamer equals the hexamer in these properties, but in the half-molecule (2 × 6-mer) cooperativity abruptly rose to 50% of the end value. The 19-meric fragment provides no further enhancement; for the jump to 100% the entire 4 × 6-mer was required (Savel *et al.*, 1986). These results are consistent with a recently introduced theoretical description of the oxygen binding process, the so-called "nesting model," an extension of the classical "MWC" model (Decker *et al.*, 1986b). This new model is based on the idea of Wyman (1984) that cooperative oxygen binding manifests itself by a hierarchy of nested allosteric units—a theoretical approach which was experimentally proved here not only for the first hemocyanin, but generally for the first allosteric macromolecule. Moreover it was interesting that the ultimate creator of all studied systemic characters is indeed the hexamer; the higher structural levels only quantitatively improve effects.

Installed subunits act as amplifiers and transmitters

We have started to characterize specific roles of the various subunits in the overall oxygen binding process, because their uniformity in the isolated state does not necessarily mean that they behave identically when incorporated in the 4 × 6 particle. Our current project deals with the analysis of reassembled 4 × 6-mers which have one chemically modified subunit type. First, we had to establish the gentle immuno affinity chromatography for subunit purification to prevent the loss of important aspects of function due to uncontrolled protein damage later in the reassembled molecules.

We have already analyzed reassembly products with incorporated "apo-subunits" (copper-free), or "met-subunits" (copper oxydized), but most of our data stem from experiments with "mercury-subunits": Mercury(II) ions undialyzably bind to tarantula hemocyanin in an amount of 1–2 atoms per subunit. The effect of a mercury treatment of the whole 4 × 6-mer is dramatic: it is still a 4 × 6-mer, and still binds oxygen, but the binding properties correspond to those of single subunits (Markl *et al.*, 1986b). This means that all subunit interactions are totally blocked. Comparable behavior is exhibited by *Limulus polyphemus* and *Callinectes sapidus* hemocyanin (Brouwer *et al.*, 1983). Correspondingly, the treatment of a subunit with mercury, followed by its reincorporation into the 4 × 6-mer, should result in an uncoupling of the interaction processes. In such experiments indeed in the case of all subunits, alterations of oxygen affinity, cooperativity, and Bohr effect were monitored (Fig. 7). According to the data, a relatively uniform role as amplifiers can be ascribed to the various monomeric subunits. Heterodimer *bc* also amplifies, and additionally seems to function as a molecular transmitter between the four hexamers (Markl *et al.*, 1986b). This is the first information about the specific contribution of individual subunit types to the allostery of any one hemocyanin molecule.

FUTURE ASPECTS

Despite our new understanding of the structure, function, and evolution of subunit diversity in arthropod hemocyanins, there remain many challenging questions. For example, tarantula hemocyanin displays its cooperativity maximum at pH 8.0, but the resting blood pH of the animal as measured *in vivo* is 7.5, and drops to 7.0 after activity (Angersbach, 1978; Loewe, 1978). In this range, however, cooperativity does not convincingly exceed the level already achieved (at least at pH 8.0) by the isolated 1 × 6 quarter-structure (Fig. 7). For what reason does a complicated, *bc*-controlled interaction between the four hexamers actually exist, if it is not, or not fully, utilized by the animal? Very recently, we discovered that the whole problem was due to our incomplete knowledge of the actual situation: calcium and magnesium ions in their native blood concentrations (4 m*M* each: Schartau and Leidescher, 1983) enhance, at pH 7.5, cooperativity to the maximum level (B. Markl and J. Markl, unpub.). It should therefore be interesting to investigate the influence of these ions, and of other modulators, on particular subunits.

Other important problems related to subunit diversity are the biological significance of changes in subunit composition while crustaceans adapt to different environments

(Mason *et al.*, 1983), the significance of ontogenetic changes, the significance of the interspecific variability of subunit beta, and similar projects. Another open field is the observation of conformational changes in certain subunits during oxygen binding, which presently is being attempted in our laboratory using fluorecent probes (Leidescher and Linzen, 1986). Although hemocyanin c-DNA already could be cloned, and sequenced (Voit and Schneider, 1986), the cytoplasmatic events in the hemocyanin synthesizing cells are still unknown. Those "cyanocytes" (Fahrenbach, 1970) proliferate in crabs from lymphocytogenic nodules in the outer gizzard wall and in spiders from the inner heart wall (Ghiretti *et al.*, 1977; Kempter, 1983). One of our most important goals is to delve into the cyanocytes, to analyze the location, structure, and organization of hemocyanin genes, which are still entirely concealed, and to reveal how the expression of the diverse subunits is regulated.

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A SCANNING ELECTRON MICROSCOPIC STUDY OF EMBRYONIC DEVELOPMENT OF A MARINE HYDROZOAN

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ABSTRACT

An indepth three-dimensional investigation examined the surface morphological changes associated with the development of a typical marine hydrozoan early cleavage embryo into a mature planula larva. During hydrozoan embryogenesis the arrangement and distribution of surface microvilli change; cilia of some epitheliomuscle cells appear before embryos gastrulate; and the embryo undergoes dramatic changes in body shape. Early cleaving embryos are bizarre in morphology, however, by the end of late cleavage the embryos have rounded to form a sphere. Gastrulation is characterized by the appearance of a blastopore at the future posterior end of the planula and by the migration of cells over the margins of the blastopore to the inside of the embryo. The product of gastrulation is a young planula which elongates and decreases in overall diameter to form a mature planula that eventually attaches via its anterior end to a substrate and undergoes metamorphosis.

INTRODUCTION

The cnidarians display a simple architecture and exhibit exceptional morphogenetic plasticity and adaptability. The phylum is unusual in that its postembryonic development has been much more thoroughly studied than its embryogenesis. Most previous investigations of cnidarian embryogenesis have concentrated on the internal morphology of the planula larva. These studies primarily utilized the techniques of light microscopy and transmission electron microscopy (Martin and Thomas, 1977, 1980, 1981a, b, 1983a; Martin and Chia, 1982; Martin *et al.*, 1983; Walch *et al.*, 1986). Hotchkiss *et al.* (1984) used a scanning electron microscopic cryofracture technique to examine both the surface morphology and the internal morphology of a hydrozoan planula and attempted to correlate their findings with other studies employing transmission electron microscopy. Although the previous work on planulae has contributed valuable information concerning the morphology of the larval form of the cnidarians, major gaps still exist in our basic knowledge of early development in this lower animal phylum. As a result of this lack of information, the following scanning electron microscopic study was undertaken to examine the surface morphological changes associated with the development of a typical marine hydrozoan early cleavage embryo into a mature planula. Such an indepth three-dimensional investigation of the cnidarian embryonic life cycle has never been done. This study provides important information concerning the external morphological changes of embryos during their development and sheds some insight into the morphogenetic shaping of the hydrozoan planula.

MATERIALS AND METHODS

Mature colonies of the marine hydrozoan *Pennaria tiarella* (McCrady) were collected from pier pilings at the North Carolina Institute of Marine Sciences, Morehead

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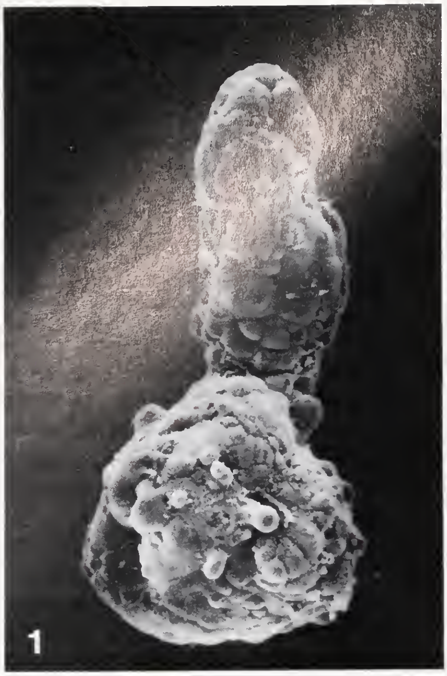
City, North Carolina. Fronds from mature male and female colonies were placed together in large finger bowls of filtered seawater. Bowls were placed in the dark at 6:00 p.m., and at 9:00 p.m. they were returned to the light. Embryos in early cleavage stages were observed in the bottoms of the dishes. These embryos were transferred to small dishes of filtered seawater and reared at 23°C to various developmental ages (Table I).

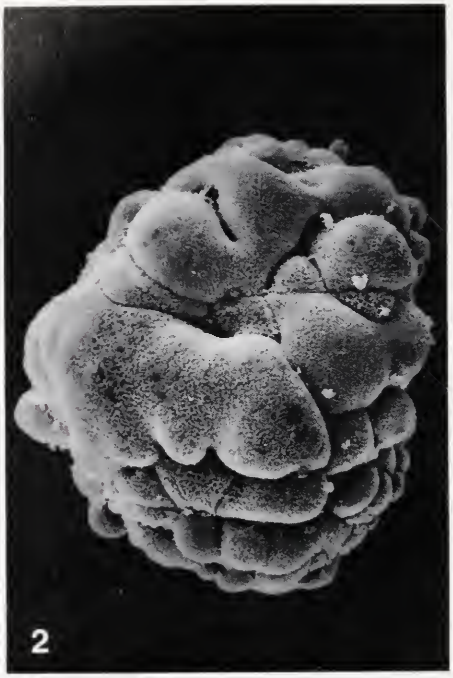
Early cleavage embryos (2–4 hours old), late cleavage embryos (6 hours old), 8-hour embryos (gastrulae), and 10-, 36-, and 48-hour planulae were prepared for scanning electron microscopy (SEM). Animals were fixed for 1 hour in 2.5% glutaraldehyde, pH 7.4, in 0.2 *M* phosphate buffer. They were postfixed for 1 hour in 2% osmium tetroxide in 1.25% sodium bicarbonate, pH 7.2. Samples were dehydrated through a graded series of ethanols and then critical point dried from CO_2 using a Denton critical point dryer equipped with a Tousimis liquid CO_2 water/particulate filter. Animals were coated with gold palladium in a Denton sputter coater. Stubs were examined with a JEOL JSM-T300 SEM operated at 15 kV. Individual cell types were identified according to developmental age of the embryo and surface specializations as previously described in transmission electron microscopic studies (Martin and Thomas, 1977, 1980, 1981a, b). Embryos undergoing gastrulation were continuously examined under a Zeiss light microscope until immature 10-hour planulae were formed.

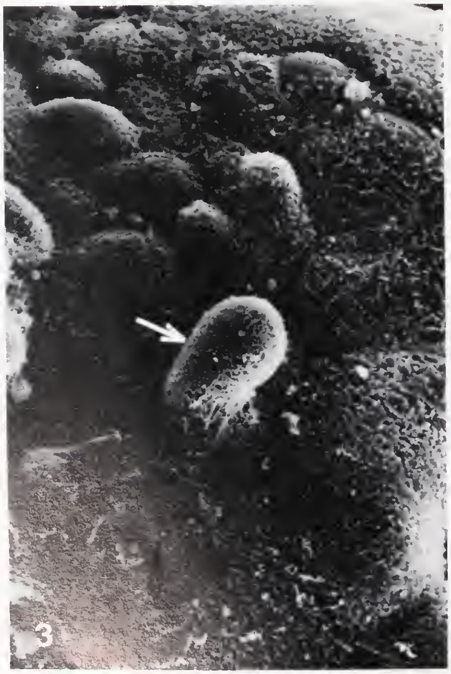
RESULTS

Cleavage in *Pennaria tiarella* embryos is holoblastic, unequal, and asynchronous (Figs. 1–4) (Martin and Thomas, 1977). The random cleavage pattern results in the formation of blastomeres of unequal size. A period of early cleavage extends from 2 hours postfertilization to the beginning of 6 hours postfertilization. During this time no one embryo cleaves in exactly the same fashion and the embryos exhibit numerous bizarre shapes and reach the 128–256 cell stage (Figs. 1, 2). Early cleavage blastomeres have numerous cytoplasmic blebs extending from their surfaces (Fig. 3). These blebs are transient and disappear in the later cleavage stages. Microvilli arranged in distinct patches project from the surface of each blastomere (Fig. 5). The tips of microvilli in each clump appear to come together at their apical ends to form a point. Early cleavage is very rapid and by 6 to 8 hours postfertilization a solid blastula (late cleavage) is formed (Fig. 6). The blastomeres are more uniform in size than during early cleavage and the embryo assumes the shape of a sphere. The embryo measures *ca.* 230 μm in diameter. During this stage a few cilia appear which are associated with the blastomeres that will give rise to the epitheliomuscle cells of the young planula (Fig. 7). By the end of 6 hours postfertilization the large blebs found in association with the early cleavage blastomeres have disappeared and a mucous-like sheath is seen covering parts of the surface of the late cleaving embryo (Figs. 6, 7). The microvilli of the late cleavage stage are shorter than those seen in earlier stages and are organized into clumps which are not as distinct as in earlier stages (Fig. 8). The microvilli are found on certain regions of the blastomere, while other regions of the same blastomere are devoid of these structures (Fig. 8). Some blastomeres possess few if any microvilli (Fig. 8).

By 8 hours postfertilization the blastomeres are of equal size. The surface of the embryo is smooth, the contours are regular, and a single indentation is present at one pole (Figs. 9, 10). This indentation corresponds to a blastopore, and the pole at which it forms corresponds to the future posterior end of the planula (Fig. 11) (Martin and Thomas, 1983b). Some of the cells at the surface appear to migrate in a radial fashion toward the blastopore, roll over the margins of the pore, and disappear to the inside







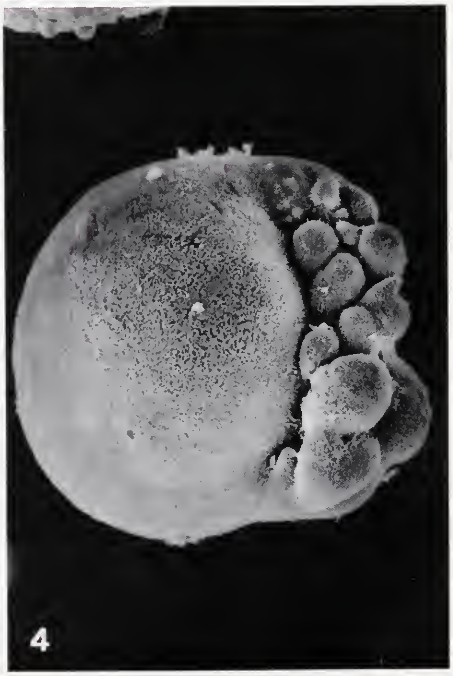
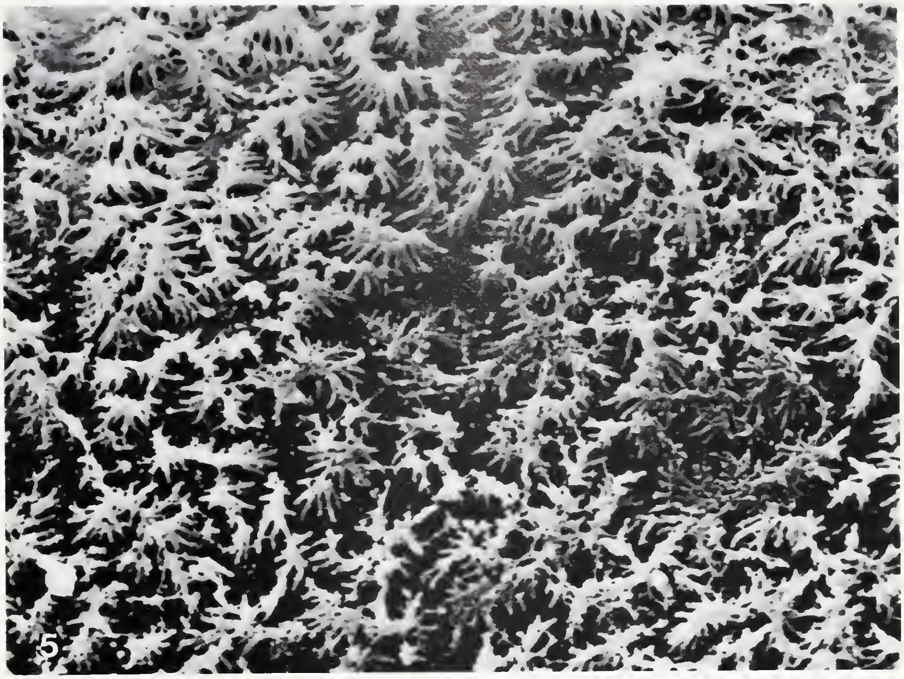


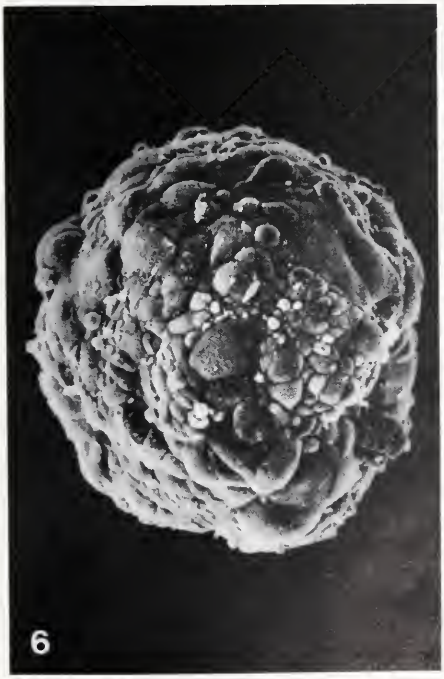
FIGURE 1. Early cleavage embryo (4 hours postfertilization). Cleavage is bizarre and unequal resulting in the formation of small and large blastomeres. ×233.

FIGURE 2. Early cleavage embryo (4 hours postfertilization). The irregular cleavage pattern in this embryo is very different from that in Figure 1. ×290.

FIGURE 3. Early cleavage blastomeres. Numerous cytoplasmic blebs (arrow) project from the early blastomeres. ×1163.

FIGURE 4. Asynchronous early cleavage embryo (2 hours postfertilization). The right side of the embryo is cleaving while the left is not. ×260.





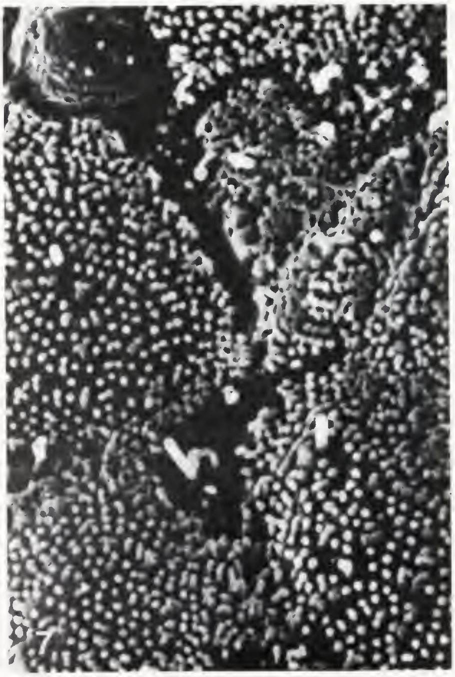
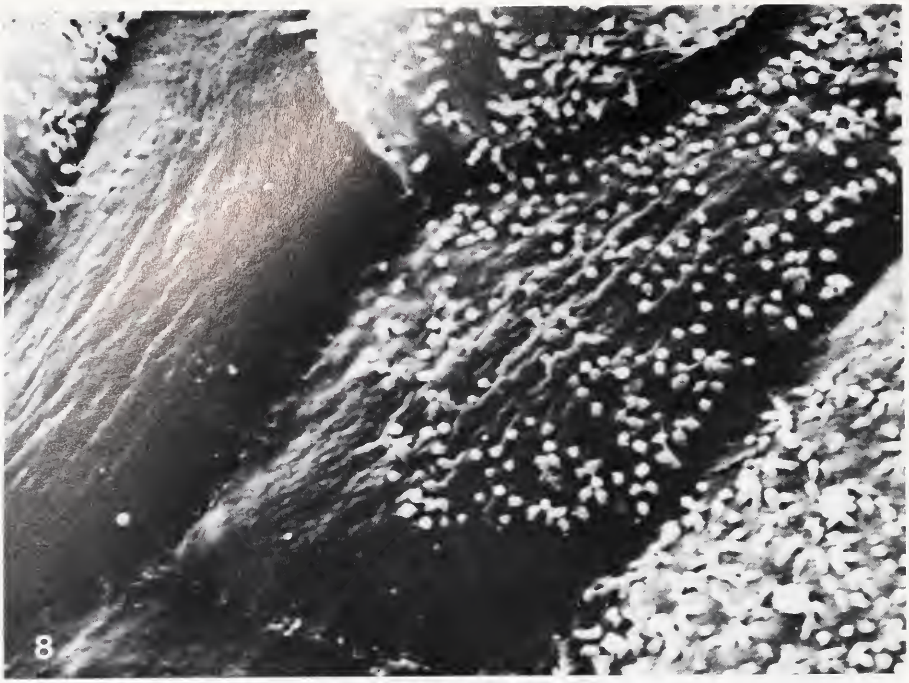
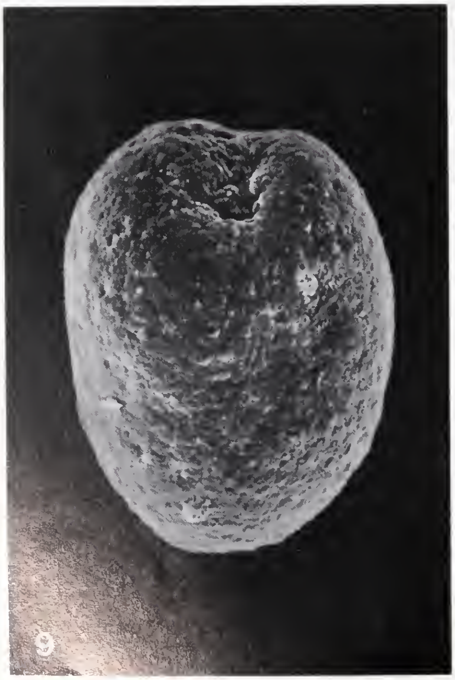


FIGURE 5. Microvilli associated with the blastomeres of an early cleavage embryo (4 hours). Each blastomere is covered with distinct patches of microvilli. The microvilli of each patch converge in their apical regions to form a common focal point. ×5425.

FIGURE 6. Late cleavage (6 hours old). During late cleavage the embryo assumes the shape of a round sphere and the blastomeres are becoming more equal in size. ×290.

FIGURE 7. Late cleavage embryo (just before gastrulation). A few short cilia and mucous appear at the surface before the animal undergoes gastrulation. ×6000.





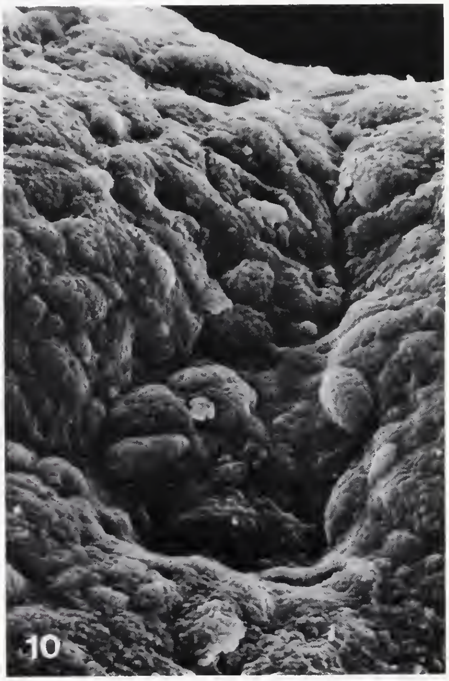


FIGURE 8. Microvilli of the late cleavage embryo (early 8 hours old). Patches of short microvilli cover certain regions of the blastomeres while other areas are bare. The microvilli associated with the patches do not come to a point in their apical regions as seen in early cleavage (Fig. 5). ×7750.

FIGURE 9. Late 8-hour gastrula. The blastomeres are relatively uniform in size and the surface is smooth. An indentation (blastopore) forms at one end of the animal. ×290.

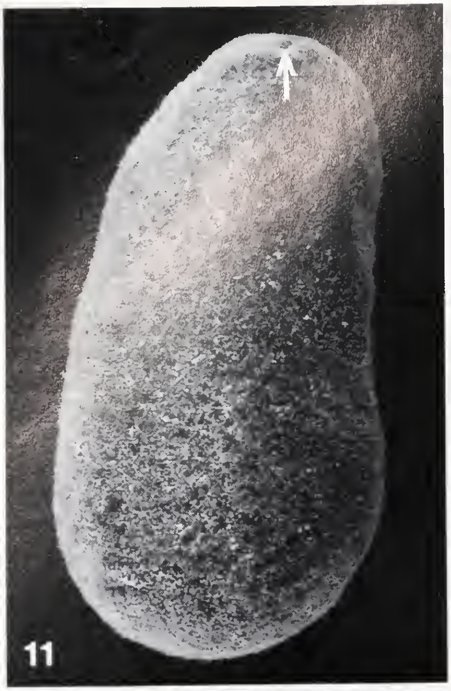
FIGURE 10. Blastopore region. Cells on the outside of the embryo move toward this pore, roll over the margins of the pore, and disappear to the inside. ×1650.

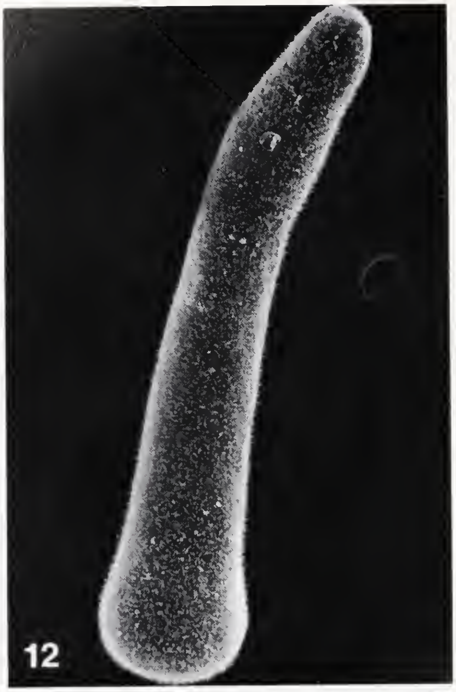
(Martin and Archer, submitted). This stage represents gastrulation. The gastrula is *ca.* 250 μm long and 190 μm wide. During gastrulation surface specializations characteristic of mucous cells appear (see Fig. 16 for gland cell specialization). These specializations consist of a single cilium surrounded by a low disorganized collar of microvilli. The cilium and microvilli sit within a small ectodermal surface depression (Fig. 16). Thus by the end of gastrulation surface projections of mucous cells and epitheliomuscle cells are distinguishable (see Figs. 15, 16). The epitheliomuscle cell possesses a single cilium that projects from its surface. These cilia are short when they first appear and later become longer as the embryo matures to the planula stage (Figs. 7, 15). Very few if any microvilli are associated with this cilium. Each epitheliomuscle cell and mucous cell is monociliated. The surface specializations of the epitheliomuscle cells and mucous cells do not change as the embryo matures, except for the elongation of the cilium. Blastomeres located closest to the blastopore possess short cilia while those farthest from the blastopore have longer cilia. Each blastomere corresponds to either an epitheliomuscle cell or a mucous cell.

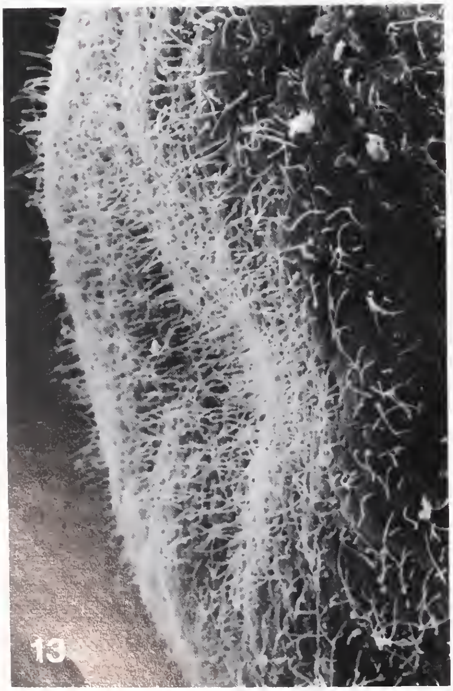
Between 8 and 10 hours postfertilization the gastrula elongates in an anterior–posterior direction to form a young planula (Fig. 11). This 10-hour planula measures *ca.* 350 μm long, 180 μm wide in the anterior region, 170 μm wide in the mid area, and 120 μm wide in the tail region. A distinct anterior pole and posterior pole are visible (Fig. 11). The blastopore is located at the posterior pole of the planula and is nearly closed (Fig. 11). The surface cells are numerous, small, and uniform in size. Epitheliomuscle cells and mucous cells comprise the ectoderm. Microvilli of the 10-hour embryo are numerous and are not arranged in distinct patches as seen during early development. They are uniformly distributed over the surfaces of the ectodermal cells. This distribution pattern persists until the planula begins to metamorphose. The 10-hour planula elongates to form the mature planula which is anywhere from 24 to 96 hours old depending upon temperature (Figs. 12, 14). During the elongation period the planula grows in length and becomes narrow in diameter; surface specializations of neurosensory cells and nematocytes appear (Figs. 17, 18); and numbers of cells increase.

By 36 hours the planula is *ca.* 700 μm long, 150 μm wide in the anterior region, 100 μm wide in the mid area, and 80 μm wide in the tail (Fig. 12). The ectoderm of the 36-hour planula consists of epitheliomuscle cells, mucous cells, and nematocytes (see Figs. 15, 16, and 18 for distinguishing surface projections of these cells). The epitheliomuscle cells comprise the majority of the ectodermal cells and are found along the entire length of the planula. Mucous cells are the second major type and are mostly concentrated in the anterior third of the animal (Martin and Archer, submitted). Numerous surface specializations of these cells are abundant in the head region. Nematocytes are located in a region extending from the anterior end of the planula to the lower third of the planula and are most abundant in the anterior end. Apical projections of nematocytes are characterized by a single cilium projecting from a basal collar of long microvilli (Fig. 18). The cilium is displaced to one side of the collar. All cilia at this stage are long (Fig. 13). By 36 hours an indentation is visible at the anterior end of the planula (Fig. 13). This indentation is not the same pore formed by the blastopore during gastrulation. Long cilia project from this indentation. This indentation appears to help the planula attach to a substrate.

At 48 hours the planula is *ca.* 800 μm long, 120 μm wide in the head region, 60 μm wide in the mid area, and 40 μm wide at the tail (Fig. 14). Five types of surface specializations are present by 48 hours (Figs. 15–18). Microvilli are scattered over all the ectodermal cell surfaces. Apical regions of epitheliomuscle cells are characterized by a solitary cilium (Fig. 15). Mucous cells possess a single cilium surrounded by a







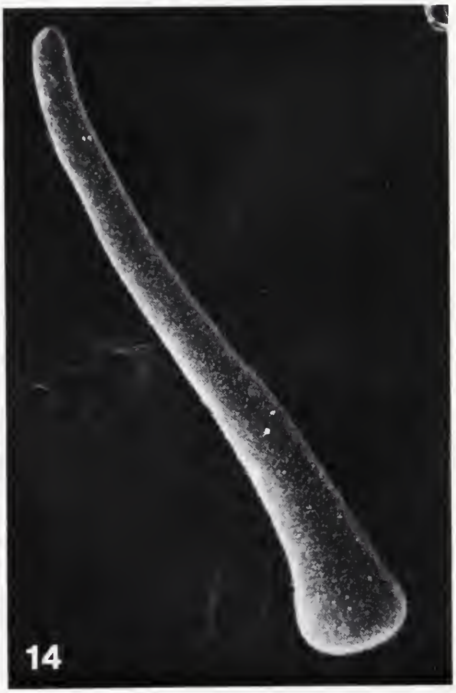


FIGURE 11. Ten-hour planula. The animal has elongated and has a distinct anterior end and posterior end. The anterior end is directed down in this micrograph. Microvilli are scattered over the surfaces of all ectodermal cells. Cilia are numerous. The blastopore (arrow) is located at the posterior tip of the planula. ×280.

FIGURE 12. Thirty-six-hour planula. The planula is long and narrow. Cilia are abundant, long, and scattered over the entire surface of the animal. The anterior and posterior regions are much narrower than seen in the 10-hour planula (Fig. 11). The anterior end is down. ×165.

FIGURE 13. Thirty-six-hour planula. An indentation is present in the middle of the anterior end. This pit is not the same indentation formed by the blastopore. Long cilia project out from the pit. ×1100.

FIGURE 14. Forty-eight-hour planula. Anterior end is directed down. ×145.

loose collar of microvilli, all of which arise from a small surface depression (Fig. 16). Apical extensions of neurosensory cells are visible by this stage (Fig. 17). These sensory cells possess a single cilium surrounded by a bulbous cluster of microvilli. The cilium is located in the center of the cluster. Neurosensory cells occur all along the length of the planula but are most concentrated in the anterior head region of the planula. Nematocyte projections identical to those described for the 36 hour planula are also seen (Fig. 18). The nematocyte specializations are more numerous than those seen at 36 hours, however, they do have the same distribution pattern. Planulae become competent to metamorphose anytime between 24 hours and 96 hours postfertilization depending on temperature. Planulae may metamorphose naturally or they can be induced to metamorphose by treating with cesium chloride (Martin and Archer, in prep.). Shortly after planulae attach to a substrate, their cells lose microvilli and cilia. For a summary of the time sequence of developmental events in embryos of *Pennaria tiarella* at 23°C see Table I.

Discussion

The planula larva is the best described representative of the cnidarian embryonic life cycle (Martin and Thomas, 1977, 1980, 1981a, b, 1983a, b; Freeman, 1981; Martin and Chia, 1982; Martin *et al.*, 1983; Berking, 1984; Walch *et al.*, 1986). The morphological events prior to planula formation have been largely ignored in the past, and as a result information on early development in the cnidarians is lacking (Martin and Thomas, 1977, 1983b; Martin *et al.*, 1983).

In an attempt to understand better embryonic morphogenesis in the cnidarians we used scanning electron microscopy to examine the development of a typical marine hydrozoan beginning with early cleavage and ending with the mature planula. During the development of *Pennaria tiarella* the embryo undergoes extensive changes in body shape. Early cleaving embryos have a bizarre morphology, however, by the end of late cleavage the embryos have rounded to form spheres. Such a rounding of the embryo may be essential if an organized form of gastrulation is to follow. In *Pennaria tiarella* gastrulation is organized with the formation of a blastopore at the future posterior end of the planula and the migration of cells over the margins of the blastopore to the inside of the embryo (Martin and Archer, in prep.). Such movements of cells resemble the morphogenetic process of invagination, a type of gastrulation not previously reported for the Hydrozoa (Tardent, 1978). A similar pattern of gastrulation

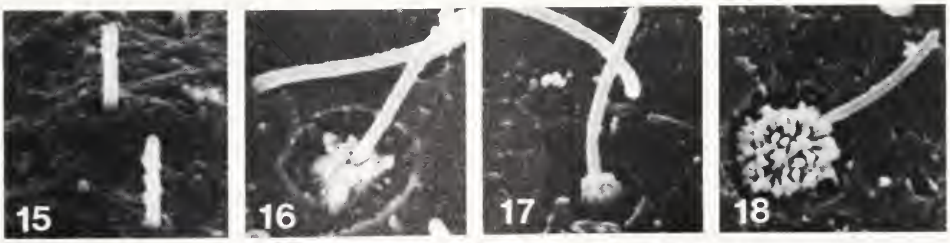


FIGURE 15. Surface projections of epitheliomuscle cells. A single cilium extends from each cell. ×6500.

FIGURE 16. Surface projection of a mucous cell. A single cilium surrounded by a disorganized clump of microvilli extends from a slight surface depression. ×6000.

FIGURE 17. Surface extension of a neurosensory cell. A single cilium projecting from the center of a collar of microvilli characterizes this cell type. ×6000.

FIGURE 18. Surface extension of a nematocyte. A single cilium projecting from the side of a collar of long microvilli distinguishes the nematocyte. ×6000.

TABLE I

Developmental time table for embryos of Pennaria tiarella

Stage	Developmental age (hours postfertilization)	Distinguishing characteristics
Early cleavage	1–6	Bizarre shape; irregular cleavage; holoblastic cleavage; asynchronous cleavage
Late cleavage	6–8	Holoblastic cleavage; more regular cleavage; more synchronous cleavage; spherical shape; cilia appear
Gastrulation	8–10	Appearance of blastopore; axial elongation; localization of embryonic tissue types; separation of germ layers with the formation of the mesoglea
Early planula	10	Anterior, posterior axis established; closure of blastopore; presence of cilia of epitheliomuscle and mucous cells
Mature planula	24–96	Axial elongation; formation of anterior depression; appearance of surface specializations of neurosensory cells and nematocytes; attachment; metamorphosis

has been observed for embryos of the hydrozoan *Podocoryne carnea*, in which a blastopore is also present (Martin and Archer, submitted). The product of gastrulation is a short fat planula. As the young animal grows into a mature planula capable of attaching and metamorphosing, planular length increases while planular diameter in the anterior, mid, and tail regions decreases.

During development of *Pennaria tiarella* the distribution patterns and numbers of microvilli and cilia change. In early and late cleavage the microvilli are abundant and are found in distinct patches. Cleavages are very rapid in these embryos and the arrangement of microvilli during this period of intense mitotic activity may play an important role in the ability of these blastomeres to adhere together and form stable contacts. The patch arrangement of microvilli may increase the surface area of these projections and hence provide sites for numerous adhesion molecules. As development proceeds through late cleavage cell division slows and the arrangement and number of microvilli change. The number of microvilli per animal increases through the mature planula stage and they are not arranged in patches. The microvilli disappear shortly after the mature planula attaches. At the beginning of gastrulation, the microvilli appear as single entities. Because cell division is slowed, pretty much synchronous, and of a more ordered pattern by gastrulation, perhaps not as many adhesion molecules are needed for the initial sticking together of these cells as were needed earlier. The presence of a single microvilli on the surfaces of cells from early gastrulation through the mature planula stage may be a reflection of the need for fewer adhesion factors during later development.

Cilia are not found on early cleavage embryos. Only after the blastomeres have rounded to form a late cleavage sphere do a few short cilia appear. These cilia are associated with blastomeres that will form differentiated epitheliomuscle cells after gastrulation. The presence of cilia on these late cleavage blastomeres may indicate that some of these early cells are predetermined during cleavage before the actual separation of the two germ layers occurs during gastrulation. As development continues to the mature planula stage cilia grow in length and increase in number. Surface specializations characteristic of mucous cells, neurosensory cells, and nematocytes form. The appearance of these specializations at particular developmental ages as determined from scanning electron microscopy corresponds well to their developmental appearance noted using transmission electron microscopy (Martin and Thomas, 1977, 1980, 1981b, 1983a).

Sexual reproduction and embryogenesis have never been a primary focal point in cnidarian research. This is surprising because the cnidaria offer excellent material for the study of the evolution of embryogenesis. Embryogenesis in the simpler forms at times appears almost "anarchic," whereas, in the more advanced cnidaria highly complex mosaic patterns are seen (Metschnikoff, 1886; Carré, 1969). The present study examines embryogenesis in a marine hydrozoan. Findings such as the arrangement and distribution of microvilli during development, the presence of cytoplasmic blebs on early blastomeres, the presence of cilia during late cleavage, the formation of a blastopore, the migration and invagination of surface cells during gastrulation, and the changes in body shape as the hydrozoan embryo progresses from early cleavage to the mature planula have not been reported previously. Additional studies of embryogenesis in the phylum are needed to fill in the major gaps that exist in our basic knowledge of morphogenesis in this lower animal phylum.

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PRECOCIOUS TERMINATION OF DIAPAUSE IN NECK- AND ABDOMEN-LIGATED PUPAL PREPARATIONS OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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ABSTRACT

When ligatures were placed between the head and thorax of freshly pupated hornworms, the resulting brainless preparations initiated adult development weeks earlier than intact diapausing controls and months earlier than similar preparations from which the brain had been surgically extirpated. This phenomenon could not be reproduced by removal of any single recognized cephalic neural or endocrine organ or any combination of organs. A similar accelerated development took place in many isolated pupal abdomens prepared by ligature or surgical section between the thorax and abdomen.

Extirpation of the prothoracic glands of either diapause or non-diapause pupae resulted in only a very slight delay in the onset of adult development. Nevertheless, this development remained highly dependent on the brain, since preparations lacking brains as well as prothoracic glands underwent a prolonged developmental arrest. Preparations lacking prothoracic glands demonstrated elevated levels of ecdysone at the outset of adult development, although these levels were slightly lower than those of intact individuals at a similar stage.

These findings suggest that sources of ecdysone outside the prothoracic glands can respond to a hormonally active brain and contribute significantly to the elevated titers of ecdysone that accompany much of normal adult development. In addition, the present results direct attention to the possible existence of a head-centered mechanism for maintenance of the low ecdysone titer necessary for the persistence of pupal diapause.

INTRODUCTION

Insect pupal diapause is classically conceived as a developmental hiatus attributable to the failure of the prothoracic glands (PG) to secrete ecdysone—a failure considered in turn to reflect the diapausing brain's inability to secrete the prothoracicotropic hormone (PTTH). But in the tobacco hornworm, *Manduca sexta,* the diapause-like arrest that ensues after surgical excision of the pupal brain is eventually terminated, albeit with substantial delay relative to intact pupae (Judy, 1972; Wilson and Larsen, 1974; Safranek and Williams, 1980). Possible mechanisms for this outcome include autonomous secretion of ecdysone by the PG or by additional tissues not subject to the classical type of regulation by the brain.

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Abbreviations: PG = prothoracic gland; PTTH = prothoracicotropic hormone; LD = long-day; SD = short-day; SEG = subesophageal ganglion; PTG = prothoracic ganglion; FG = frontal ganglion; MTG = mesothoracic ganglion; VNC = ventral nerve cord segment; CC-CA = corpora cardiaca-corpora allata complexes.

For forty years the PG have been considered the principal source of ecdysone. Nevertheless, ecdysteroid production in at least certain species has been shown to proceed outside the PG in sources that include the testes, ovaries, and oenocytes (for reviews, see Hoffman and Hetru, 1983; Rees, 1985). The physiological significance of these abdominal sources is supported by instances in which ecdysone-dependent development takes place in the absence of the PG–for example, after surgical extirpation of the PG or in isolated abdomens (Chadwick, 1955; Ichikawa, 1962; Hsaio *et al.,* 1975; Delbecque *et al.,* 1978; Delbecque and Sláma, 1980; Sláma, 1983). But most studies to date fail to clarify whether these sources contribute significantly to the rising levels of ecdysone that accompany molting in intact insects.

The brain is known to be an important and sometimes necessary organ for the generation of molt-inducing levels of ecdysone. But again, numerous instances of ecdysone-dependent development in the absence of the brain have been described (see Safranek and Williams, 1980, for review). The current study focuses on the pupal stage of the tobacco hornworm where we have encountered the paradoxical finding that decapitation of freshly pupated diapause-destined individuals actually accelerates the initiation of adult development. The present experiments document this finding and probe its endocrine basis, drawing attention to previously unsuspected aspects of the regulation of normal adult development.

MATERIALS AND METHODS

Hornworms were reared as described previously (Safranek and Williams, 1980) at 25°C under either a short-day (SD, 12L:12D) or long-day (LD, 17L:7D) photoperiod. SD pupae were derived from larvae reared under SD conditions and normally underwent a pupal diapause. LD pupae were derived from larvae under LD conditions; these did not diapause but initiated development typically within five days after pupation. The day of pupation is termed "Day 1" of the pupal stage, and the first seven days of the pupal stage "Week 1." Operations and ligations were carried out as described previously (Safranek and Williams, 1980; Safranek *et al.,* 1980) and as described below. All operated preparations were maintained under SD conditions at 25°C, the initiation of development being recognized by detachment and retraction of the trachea from the overlying pupal cuticle of the forewings.

Removal of the subesophageal ganglion (SEG) or the prothoracic or mesothoracic ganglia (PTG, MTG) was accomplished through a small ventral midline incision beginning at the base of the pupal proboscis; in many instances the proboscis was still quite flexible and could be bent slightly from the midline to facilitate the surgical approach. Removal of the prothoracic glands (PG) was accomplished after removal of a rectangular section of the dorsal thoracic cuticle. The main body of each PG was identified nested in its characteristic position just medial to the large tracheal trunk adjoining the prothoracic spiracle. It was gently teased free of its fine connections to surrounding tissues and then withdrawn by the aid of two forceps used in a hand over hand fashion to draw the remainder of the gland slowly from more anterior regions. Abdomens were prepared by ligation within 6 h of larval-pupal ecdysis shortly after the pupal wings attained their final size and position. Older abdomens were surgically isolated using a razor blade to section the pupal cuticle and body wall behind the thorax. The gut was either ligated with a fine sterile thread or draped over the side of the exposed edge of the abdomen. A sterile glass cover slip was applied over the anterior end of the abdomen and sealed with melted wax (Tackiwax, CENCO). Radioimmunoassays of ecdysteroids were performed as described elsewhere (Carrow *et al.,* 1981).

RESULTS

Effects of brain removal and head-ligation on the development of pupae

In the course of studies on the regulation of pupal diapause we noted that pupae head-ligated promptly after eclosion to the pupal stage initiated development much sooner than would have been expected of brainless pupae. To document this we ligated 100 SD pupae within 2–6 h after pupation. An additional 100 SD pupae were set aside as unoperated controls. All individuals were examined at weekly intervals for the onset of tracheal apolysis. As documented in Figure 1, the ligated preparations initiated development an average of one month prior to intact diapausing pupae and approximately four months earlier than expected for pupae whose brains had been surgically extirpated (Safranek and Williams, 1980).

We repeated the experiment on approximately 50 freshly ecdysed SD pupae and 50 similar LD pupae, with 50 intact SD pupae serving as controls. Here again over 50% of the neck-ligated preparations had initiated adult development by the fifth week, irrespective of LD or SD status; by the ninth week over 90% had done so. By contrast, 50% of the intact SD controls initiated development only after 11 weeks and 90% only after 13 weeks. The development of the LD preparations thus was delayed by more than a month relative to that of intact LD pupae, which, as previously mentioned, initiate development within a week of pupation. But both LD and SD preparations developed in this instance over a month before intact diapausing pupae and several months earlier than would have been expected of pupae whose brains had been excised rather than removed by neck-ligation.

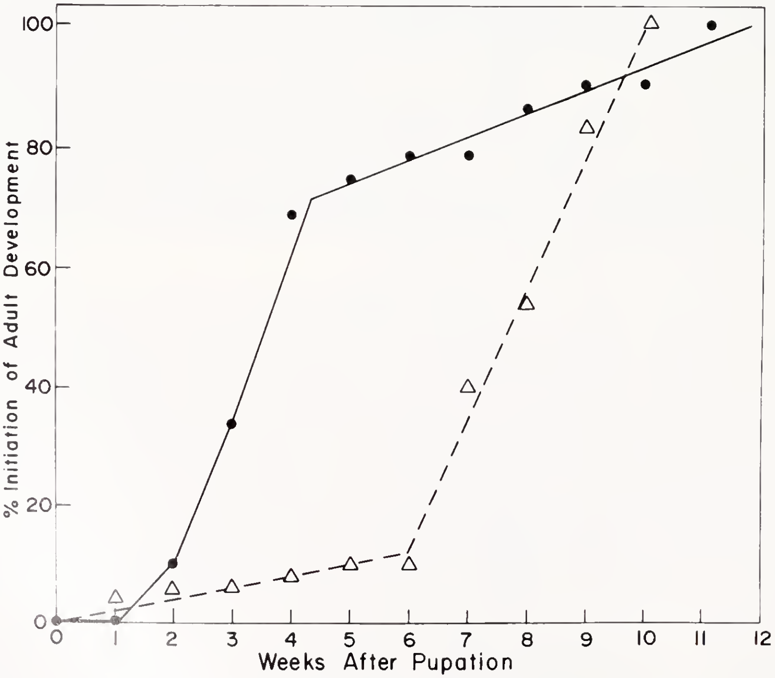

FIGURE 1. Acceleration of diapause termination by neck-ligation of diapause pupae. Diapause pupae were ligated 2–6 h after ecdysis. Initiation of development was recognized by apolysis of the wing epidermis. Ligated preparations are indicated by dark circles, intact controls by clear triangles.

Two explanations for these results seemed possible. Either a developmental inhibitor existed in the head which could be removed by ligation but not by brain excision or else the ligation itself provided an ecdysiotropic stimulus to the headless preparations. We examined these possibilities in the following experiments.

Effects of removal of the brain plus other cephalic organs

We attempted to identify a cephalic inhibitor by surgical extirpation of known cephalic neuroendocrine centers. Because head-ligated preparations lacked the brain as well as all other cephalic organs and because surgical extirpation of the brain alone produced a greatly prolonged diapause that could be readily distinguished from the abbreviated diapause of head-ligated preparations, most of our experiments involved simultaneous removal of the brain in addition to one or more of the other cephalic organs. Thus, the brain was removed in tandem with the subesophageal ganglion (SEG), the frontal ganglion (FG), the prothoracic ganglion (PTG), the mesothoracic ganglion (MTG), or the corpora cardiaca-corpora allata complexes (CC-CA). In addition, brain removal was performed in combination either with severance of all neural connections to the SEG, which was thereby left wholly free in the head, or with severance of the neural connection between the SEG and PTG only. Additional groups of brainless pupae with appropriate sham operations were established as controls. These experiments included operations on the PTG and MTG because of the proximity of these ganglia to a neck-ligature. All these experiments were performed on diapause-destined SD pupae 6 to 12 h after pupal ecdysis.

The results (Table I) demonstrated no significant differences between the various groups of pupae. Irrespective of the surgical procedures performed, all groups entered an extended diapause averaging over 30 weeks.

We considered in addition the possibility that the brain itself might be the source of an inhibitor. We were driven to entertain this possibility because for reasons of technical ease we routinely head-ligated pupae about 2 to 6 h after pupal ecdysis but normally surgically removed the brain 6 to 12 h after ecdysis. Thus, the brain could potentially have released an inhibitor during the initial 6 h after ecdysis. To investigate this possibility we removed the brains from a group of larvae 1–2 days prior to the wandering period; as controls we sham-operated additional larvae. Subsequently late on the day of pupal ecdysis we removed the brains of half of the previously sham-operated individuals. In addition, we sham-operated all the other individuals, both those that had previously lost their brains and also the other half of the previously sham-operated group. We subsequently monitored all the preparations for the initiation of development at weekly intervals. All of the 23 twice sham-operated preparations initiated adult development after 1–4 months. By contrast, brainless pupae entered an extended diapause regardless of whether the brain had been removed before ($n = 17$) or after pupation ($n = 23$). At 4 months less than 25% of either group had initiated development, and after 7 months more than 50% of each group still remained in diapause. Thus, evidence for a brain-derived inhibitor was entirely negative.

Effects of PG removal on adult development

All these results failed to define an inhibitory center in the head. This raised the possibility that head-ligation itself might produce an injury reaction which elicited development through a stimulatory effect on the PG. To examine this possibility we implanted into brainless, diapausing 1-day-old pupae the PG removed from 3-day-old SD pupal preparations that had previously been head-ligated on Day 1. As controls we implanted the PG from intact Day 3 SD pupae into brainless 1-day-old pupal hosts and thereafter examined all the preparations for development at weekly intervals. During the subsequent 6 weeks, only 1 of the 15 pupae in each group initiated development. This contrasted greatly with the rapid development of a further control group of 18 head-ligated preparations in which half had initiated development by 6 weeks. These findings indicated that head-ligation did not generate within three days of ligation any irreversible, significant increase in PG activity.

In the preceding experiment, after removal of the PG from the head-ligated pupae, the thorax of each was resealed and the preparation observed at weekly intervals for the initiation of development. Much to our surprise, extirpation of the PG failed to block development of these decapitated preparations. At six weeks similar percentages of head-ligated pupae (9 of 18) and head-ligated pupae lacking PG (8 of 15) had terminated diapause, suggesting that development of head-ligated pupae did not require ecdysone secretion by the PG.

We initially considered that our surgical procedure must not have removed the entire PG. But repeated, detailed dissection of pupae after PG extirpation failed to reveal any retained portion of the PG. Comparison of the extirpated PG with whole PG carefully dissected from sacrificed pupae did not suggest that our routine operation left behind any portion of the PG.

These concerns were rendered moot by additional experiments. We prepared over 300 isolated pupal abdomens either by ligation within 4 h of the larval-pupal ecdysis or by surgical isolation of slightly older pupal abdomens as described under Materials and Methods. Although abdomens prepared by either approach had a very low mortality within the first week, preparations of both types experienced a sharply increasing mortality thereafter. Few preparations exhibited spontaneous motion beyond eight

TABLE I

Effects of surgical extirpation of cephalic and thoracic organs on diapause termination

Organs removed[1]	Number of preparations	% Developed at 50 weeks	Average time to development[2]
Brain plus sham[3]	181	67	35 ± 12
Brain plus SEG	41	85	34 ± 10
Brain plus PTG	27	81	31 ± 10
Brain plus FG	21	38	33 ± 12
Brain plus MTG	11	73	32 ± 11
Brain (SEG loose)	22	73	34 ± 12
Brain plus VNC[4]	17	65	39 ± 12
Brain plus CC-CA	16	78	36 ± 10

[1] Abbreviations: SEG: = subesophageal ganglion; PTG = prothoracic ganglion; FG = frontal ganglion; MTG = mesothoracic ganglion; VNC = ventral nerve cord; CC-CA = corpora cardiaca-corpora allata complexes.

[2] Averages are ± standard deviation and are calculated only for those preparations which had developed by 50 weeks. Thus the average time to development for the entire group of preparations would have been longer than that listed here.

[3] A group of sham-operated pupae was established for each experimental group of preparations listed below. No experimental group developed at a rate significantly different from its control group or from the pooled set of sham-operated preparations as listed on this line. Shams included brain removal plus a sham operation on the ventral thorax except in the control group for preparations without the brain and CC-CA complex, in which instance only the brain of the sham preparations was removed through a cephalic incision and no ventral incision was placed.

[4] The segment of the ventral nerve cord between the SEG and PTG was severed.

weeks. Nevertheless we repeatedly witnessed the spontaneous development of both male and female abdomens prepared by either technique: the earliest fully scaled and pigmented adult abdomen was obtained five weeks after pupation, others after as long as six months. Overall we have witnessed the adult development of 27 pupal abdomens, with approximately equal numbers of males and females and an average time from the isolation of the abdomens to the completion of adult development of 12 weeks. If survival could be enhanced, our experience suggests that many more, and perhaps all, of these preparations would be able to undergo adult development. The time course of development of these abdomens suggested that an abdominal source of a molting hormone could account for the spontaneous development of head-ligated pupal preparations: although we could not witness the initiation of adult development in isolated abdomens, the first individuals to complete adult development among either the head-ligated preparations or the isolated abdomens did so after about five weeks. Development took place in abdomens isolated from both LD and SD pupae; although the sample size was relatively small, no differences were noted in the times at which development was completed within the two groups.

What might this unusual source of molting hormone contribute to normal development? As we have seen here and elsewhere (Safranek and Williams, 1980), surgical extirpation of either the LD or SD pupal brain results in a developmental arrest of several months duration. By contrast, extirpation of the PG fails to block development of otherwise intact pupae. This was demonstrated in the case of LD pupae after removal of the PG from 12 Day 2 pupae: all of these pupae subsequently initiated development within 6 weeks of the operation, the earliest at 1 week, and 75% by 2.5 weeks. This represented a delay of only about 1 week relative to 12 control pupae whose PG had been extirpated and then replaced into the thoracic cavity, all of which initiated adult development within 6–11 days of the operation. PG removal also failed substantially to alter the duration of diapause in SD pupae. In this instance PG were removed from 18 Day 3 SD pupae; 15 similar pupae served as controls after receiving sham operations. Half of the 15 controls had initiated development at 9 weeks, as had half of the preparations lacking PG. The controls had an average diapause duration of 12 weeks, the experimentals, 13 weeks. Neither preparations subjected to PG extirpation nor sham-operated controls eclosed successfully; nevertheless, the course of adult development appeared grossly similar for both groups and essentially normal in its character and duration.

These experiments demonstrated the ability of an ecdysteroid source outside the PG to initiate and support adult development. In addition, the more rapid development of LD preparations relative to their SD counterparts suggested that these sources could respond to a hormonally active brain. Further to demonstrate this phenomenon we implanted brains from Day 1 LD pupae into Day 3 SD pupae from half of which both the brain-CC-CA complex and PG were removed, from the other half, the brain-CC-CA complex only. Both groups developed promptly. Of 10 preparations that had retained their PG, all commenced development within 6 weeks, the average being 2 weeks. Among the group without PG 3 of 14 developed only after several months whereas the remaining 11 initiated development within 5 weeks, the average of these being 3 weeks. Manifestly, the absence of the PG failed to block or even to delay substantially the rapid onset of development in response to a LD brain. In an additional control group of 15, the brain-CC-CA complex and the PG were removed but a LD brain was not implanted. All of these preparations underwent a prolonged diapause of at least three months and more than half remained in diapause after six months. As indicated in the previous experiments, these data again demonstrate that development can be stimulated by a hormonally active brain even in the absence of PG.

Is the development of preparations lacking PG accompanied by elevated ecdysteroid titers?

We inquired whether the onset of adult development in pupae lacking PG was accompanied by a rise in the ecdysteroid level. To this end we collected hemolymph samples from 3 groups of pupae on the day of tracheal apolysis—namely, intact LD pupae, LD pupae whose PG had been removed 24–48 h after pupation, and SD pupae whose PG had been removed at 2 weeks after pupation and which at that time had also received an implantation of a Day 1 LD pupal brain. We also measured the ecdysteroid levels of a group of 2–4 week old diapausing SD pupae. Samples were analyzed in a RIA for ecdysteroids. The results are summarized in Table II. Manifestly, both sets of preparations lacking PG had ecdysteroid levels markedly greater than those typically found in the course of diapause. Nevertheless, these titers were only about one-fourth those occurring in the intact LD pupae at a similar developmental stage.

DISCUSSION

The present results describe a peculiar abbreviation of the hornworm pupal diapause when head-ligation rather than surgical excision was employed for removal of the brain promptly after pupal ecdysis. Adult development was advanced by months relative to surgically debrained preparations and by weeks relative to intact diapausing pupae. Yet we were unable to identify a cephalic source of a molting inhibitor among recognized neural or endocrine organs in the head.

We nevertheless continue to favor an inhibitor as an explanation of this phenomenon. Although we cannot rule out the involvement of a powerful stimulatory injury effect as can sometimes be seen after surgical manipulation of mature pupae (Wilson and Larsen, 1974; pers. obs.), several arguments oppose this explanation and favor an inhibitor. First, the considerable surgical injuries resulting from combined brain and SEG-PTG removal had no stimulatory effect on development, nor did we find evidence of any irreversible activation of the PG after ligation. Indeed, our experience suggests that injury shortly after pupation delays development: this is the case with young non-diapausing pupae where even a small cephalic cuticular wound will delay development by 1–2 days. Second, the developmental stimulation witnessed following neck-ligation of pupae does not occur promptly as one might expect from injury; rather the stimulation becomes apparent only after at least two weeks and often much longer. Third, the active involvement of an inhibitor during diapause would account for the ability of hornworm pupae to develop in the absence of the brain: the eventual disappearance of the hypothetical inhibitor would permit resumption of development even in the absence of a positive stimulus from the brain. These considerations lead

TABLE II

Effects of prothoracic gland extirpation on the ecdysteroid titer at the outset of adult development

Procedure	Stage	Ecdysteroid titer[1]
SD pupa-PG + LD brain	Tracheal apolysis	5.9 ± 1.9
LD pupa-PG	Tracheal apolysis	4.9 ± 1.8
LD pupa, intact	Tracheal apolysis	22±3
SD pupa, intact	Pupal diapause, 2 weeks	0.21 ± 0.03

[1] The ecdysteroid titer is expressed in μg/ml β-ecdysone equivalents.

us to suggest that in the normal course of the hornworm's pupal diapause an inhibitor is elaborated by the cephalic region that is responsible for the maintenance of the diapausing condition. Over time this inhibitor is eliminated by breakdown and/or by cessation of its production, thereby permitting the initiation of adult development. This model, consistent with the present findings, finds some support in the literature.

Results similar to those recorded here have been noted in *Antherea pernyi* (Waku, 1959) wherein pupae head-ligated promptly after pupation experienced an abbreviated diapause relative to both brainless preparations and intact unchilled diapause pupae. So also, in *Pieris brassicae* removal of the brain during diapause accelerated diapause termination; moreover, abdomens isolated from chilled diapausing pupae underwent spontaneous development in contrast to the persistent diapause of abdomens isolated from young, unchilled individuals (Kono, 1977). The acknowledged preeminent role of a molt-inhibiting hormone in the development of Crustacea illustrates the potential for inhibitory control of the ecdysteroid titer among arthropods (Highnam and Hill, 1977). These examples, as well as our own, do not preclude a contributing role for the brain in diapause termination. Indeed, we suggest that pupal-adult development may reflect an interplay of both stimulatory and inhibitory factors.

The final noteworthy finding of our study is the ability of isolated pupal abdomens to initiate adult development. This propensity is unique to this stage, since isolated larval abdomens, even when implanted with brains, fail to undergo a molt (Safranek and Williams, 1980). Development of isolated abdomens or of preparations lacking prothoracic glands has previously been noted in late larval and pupal stages of diverse orders of insects (Chadwick, 1955; Hsiao *et al.*, 1975; Delbecque *et al.*, 1978; Delbecque and Sláma, 1980) including the Lepidoptera (Ichikawa, 1962; Kono, 1977; Sláma, 1983). Production of ecdysteroids outside the PG has been even more widely documented and has been shown to derive from testes (Loeb *et al.*, 1982), ovaries (see Hoffman and Hetru, 1983, for review), or oenocytes (Romer *et al.*, 1974; Studinger and Willig, 1975). We do not presently know the source of molting hormone driving the development of isolated pupal hornworm abdomens, but since this development occurred in both male and female abdomens on a similar time scale, we favor an extra-gonadal source.

The contribution of these abdominal sources to normal adult development is uncertain. The present experiments make clear that sources of ecdysteroids outside the PG are more active in the presence of a non-diapausing brain. Moreover, in either diapause or non-diapause pupae these sources can support development in the absence of PG at a rate very nearly that of intact pupae. When development ensues in the absence of PG, ecdysteroid levels rise into a range near that of intact pupae. We note that development of preparations without PG does typically lag that of controls by several days and that the ecdysteroid titers at the outset of tracheal apolysis are uniformly slightly lower than those of controls at the same stage of development. Nevertheless the extra-PG sources clearly respond to an active brain and can generate high developmentally effective levels of ecdysteroids at least 25-fold greater than those found during the course of diapause. Thus, our observations indicate that at least one novel source of ecdysone plays a definite and possibly important role in the endocrine events normally associated with the onset of adult development.

Although the ecdysteroid source outside the PG responds to an active brain, it may differ from the PG in other aspects of its regulation. We note in particular that the time courses of development initiation in head-ligated preparations with or without PG as well as of isolated abdomens all appeared quite similar. Thus, the peculiar accelerated development of neck-ligated diapausing pupae could potentially reflect the activity largely of an abdominal ecdysteroid source. This source might be especially responsive to the disappearance of an inhibitory influence emanating from the head.

Manifestly the picture presented here differs substantially from that derived through classic studies of the Cecropia silkworm. Whether one or the other picture will prove paradigmatic we cannot yet say. But certainly the literature portrays a complex picture even among the silkworms. We have already noted the work of Waku in *Antherea pernyi.* In addition, Ichikawa (1962) described the development of isolated abdomens of *Samia cynthia* in the presence of an active brain. And the regulation of diapause termination in unchilled or inadequately chilled Cecropia or in Cecropia that fail to develop promptly after termination of chilling but which ultimately develop all remain to be investigated. Although the present experiments along with those of others make clear that the regulation of pupal diapause termination in the Lepidoptera will very likely not make for a simple yarn, even now we see evidence of a common thread.

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PARTIAL PHOTOPERIODIC CONTROL OF DIAPAUSE IN THREE POPULATIONS OF THE FRESHWATER COPEPOD *DIAPTOMUS SANGUINEUS*

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ABSTRACT

Populations of the freshwater calanoid copepod *Diaptomus sanguineus* inhabiting three Rhode Island ponds switch from making subitaneous (immediately hatching) to diapausing eggs on different dates. From results of previous research the timing of diapause appears to correspond closely to the individual causes of seasonally harsh conditions in each pond. The results of rearing copepods from each pond in controlled laboratory environments indicate that each population possesses a unique spectrum of sensitivity to photoperiod. The responses obtained, however, fail to describe adequately either the rapidity with which the onset of diapause occurs in natural populations, or the differences in diapause timing between ponds. In initiating diapause, the copepods must respond to seasonal environmental cues other than critical photoperiod.

INTRODUCTION

Diapause is a physiological and developmental response adopted by a broad variety of animals as a means of avoiding seasonally uninhabitable periods in the environment. Annual climatic changes are frequently responsible for the onset of harsh conditions, and examples of temperate zone animals that enter diapause prior to winter are especially well documented (see Danilevskii, 1965; Beck, 1980; and Tauber *et al.,* 1985 for reviews). When a species' geographical range covers a broad spectrum of latitudes, intolerable winter conditions for local populations may begin at markedly different times of year. Often these populations exhibit responses to diapause-controlling cues correlated with the climatic gradient. For example, animals collected at northern latitudes initiate diapause earlier in the autumn or at longer photoperiods than those collected further south (*e.g.,* Showers *et al.,* 1975; Holtzer *et al.,* 1976; Lumme and Oikarinen, 1977; Marcus, 1984). Several investigators have shown that such variation is genetically based indicating that populations are specifically adapted to the latitudes at which they reside (Masaki, 1963, 1967; Showers *et al.,* 1975; Istock, 1981; Marcus, 1984). At the same time, there is evidence that some local populations retain additive genetic variance for diapause traits (Hoy, 1977; Istock, 1981; Lumme, 1982; Hairston and Walton, 1986), presumably maintained by local variation in selection pressures.

A third potential source of variation in diapause is that between populations residing at the same latitude. For such variation to exist, however, local habitats must be sufficiently isolated or represent sufficiently different selective regimes to overcome the homogenizing force of dispersal. The planktonic freshwater copepod *Diaptomus sanguineus* is a resident of small lakes and ponds in northeastern North America

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(Wilson, 1959). Within any given geographical area, many of these bodies of water differ in potentially important ways such as basin depth with its accompanying effect on the annual water-temperature cycle, seasonal permanency, and the types of planktivores present.

Three populations of *D. sanguineus* residing in three ponds in southern Rhode Island exhibit distinct seasonal patterns of egg diapause (Fig. 1A). In Bullhead Pond the copepods switch from production of subitaneous (immediately hatching) eggs to production of diapausing eggs in late March; in Pond C diapause is initiated at the end of April, and in Pond A diapause begins principally in mid-May (Hairston and Munns, 1984; Hairston *et al.*, 1985; Hairston, 1986). These patterns are regularly repeated in successive years and represent real differences between reproductive phenologies of the three populations even though the ponds in which they live are geographically quite close to each other (Ponds A and C are <200 m apart and *ca.* 27 km from Bullhead Pond). We showed previously (Hairston and Olds, 1984) that female copepods reciprocally transplanted between Bullhead Pond and Pond A began production of diapausing eggs at the time of year appropriate to their home pond rather than the pond to which they were transferred, and concluded that *D. sanguineus* was physiologically unable to alter its reproduction in response to changes in pond type. What then is the basis for the differences in timing of diapause observed between ponds?

One obvious possibility is that the copepods have distinct adaptations suitable to the local habitat in which they live. Hairston and Munns (1984) showed that *D. sanguineus* in Bullhead Pond began production of diapausing eggs at the appropriate time to avoid a spring increase in planktivory by resident sunfish. Ponds A and C, on the other hand, are temporary bodies of water and contain no fish. In Pond C the copepods switch to making diapausing eggs at the correct time of year to avoid seasonally intense predation by the dipteran larvae *Chaoborus* and *Mochlonyx* (Hairston *et al.*, 1985; Hairston, 1986). Neither of these predatory flies is abundant in Pond A, where *D. sanguineus* appears to produce diapausing eggs as an adaptation to survive periods of pond drying (Hairston *et al.*, 1985; Hairston and Olds, in prep.). If timing of diapause is a distinct adaptation of the copepods to the separate conditions found in each pond, then the differing seasonal patterns of reproduction must have a genetic basis. Photoperiod is the environmental cue initiating diapause in a broad variety of animals including insects (*e.g.*, Beck, 1980; Tauber and Tauber, 1981), mammals (Flint *et al.*, 1981), and crustacean zooplankton (Einsle, 1964; Stross and Hill, 1965; Stross, 1969; Spindler, 1971; Watson and Smallman, 1971; Marcus, 1980). As a result, it is logical to ask if the different *D. sanguineus* populations switch from production of subitateous eggs to diapausing eggs at different critical day lengths.

The data in Figure 1A can be replotted as the fraction of each population carrying subitaneous eggs (as opposed to diapausing eggs) at the day length prevailing when the copepods were collected (Fig. 1B). If the interpopulation differences in the timing of diapause initiation result from the copepods being sensitive to different critical day lengths, then animals collected at each of the three ponds and reared in the laboratory under a series of appropriately chosen photoperiods should respond with distinct patterns of subitaneous and diapausing egg production. Specifically, at day lengths shorter than about 11 hours all copepods should make subitaneous eggs, at intermediate day lengths of around 13 hours Bullhead Pond copepods should make diapausing eggs while copepods from Ponds A and C should make subitaneous eggs, and at day lengths longer than about 14.5 hours all copepods should make diapausing eggs. Here we test this hypothesis as a route to understanding the causes underlying the distinct reproductive phenologies of *D. sanguineus* living in Bullhead Pond, Pond A, and Pond C.

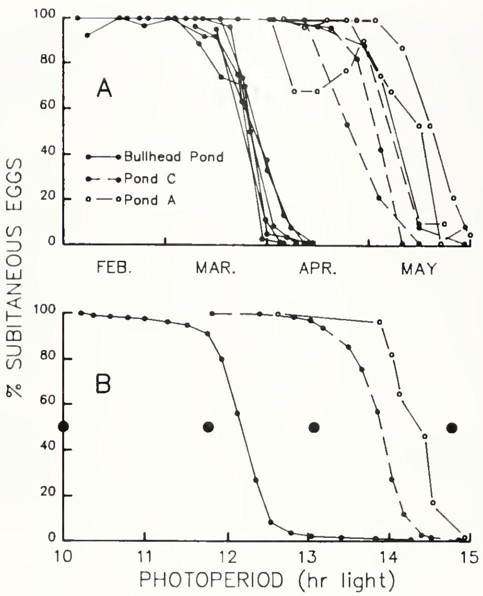

FIGURE 1. The fractions of female *Diaptomus sanguineus* from three Rhode Island ponds making subitaneous (immediately hatching) eggs, as opposed to diapausing eggs, as a function of time of year. A. Egg type *versus* date of collection. The lines are summaries of data from Hairston and Munns (1984), Hairston *et al.* (1985), and Hairston (1986) showing the annual variation in timing of diapause. Data points after the switch to production of diapausing eggs are omitted here to facilitate illustration. Copepods in temporary Pond A have been shown in some years to return to production of subitaneous eggs for brief periods in June after the pond refilled with water from heavy rains. These reversals, discussed in detail elsewhere (Hairston and Olds, 1984; in prep.), have been omitted here to simplify illustration. B. Egg type versus prevailing day length (sunrise to sunset) on the date of collection. Photoperiodic responses are calculated from mean fractions of females making subitaneous eggs for the years illustrated in A. Closed circles denote photoperiods tested in laboratory experiments.

MATERIALS AND METHODS

The study sites, Bullhead Pond and Ponds A and C, are described in detail elsewhere (Hairston, 1980; Hairston *et al.*, 1983; Hairston and Olds, 1984). *Diaptomus sanguineus* has 12 separate instars: six naupliar and six copepodid stages, plus an egg stage which the female copepods carry in a sac attached to their urosome. The animals are active in the water column during winter and spring, and make two or three generations per year before producing diapausing eggs (Hairston and Olds, 1984; Hairston *et al.*, 1985). Live first and second stage nauplii were collected during March through May from each of the three Rhode Island ponds using a 75 μm mesh net. The nauplii were isolated in 2 L glass jars filled with 1 L of filtered pond water; 200 nauplii per jar. These cultures were fed twice weekly from algal stocks, and maintained with approximately 1×10^7 cells $\cdot$ ml^{-1} of *Chlamydomonas* sp. and 1×10^5 cells $\cdot$ ml^{-1} of *Euglena* sp. The copepods were reared at 9°C and at a series of photoperiods in controlled environment chambers. Survival under these laboratory conditions was 40% to 60% from nauplius to adult. Raising the animals from early naupliar instars ensured more than sufficient time for the copepods to respond to the photoperiod treatments. Elsewhere we have shown that upon exposure to a change of day length, female *D. sanguineus* can alter the type of egg they produce within three clutches (Hairston and

Olds, in prep.). Thus, unlike many species of insects (Beck, 1980), there is no early sensitive instar that irreversibly programs the copepod's diapause phenology. It seems likely that egg type in *D. sanguineus* is determined principally by females because diapausing eggs have a thick, highly structured chorion laid down by the mother whereas subitaneous eggs have a thin homogeneous chorion, and because the two egg types are provisioned differently (Hairston and Olds, 1984). Probably due to these structural differences and the physiological adjustments they imply, clutches are composed of either subitaneous or diapausing eggs. Mixed clutches are never made. Females do not store sperm and mating is required before each clutch can be produced. We do not know, however, what role, if any, males play in determining egg type.

The photoperiods were chosen to conform with the design described in the Introduction (Fig. 1B). Day length 9:55 corresponds to the date 25 January (sunrise to sunset), well before the switch to diapause in any of the populations. Day length 13:05 corresponds to 10 April when the Bullhead Pond population has almost completely shifted to making diapausing eggs but the Ponds A and C populations still produce principally subitaneous eggs, and 14:45 corresponds to 28 May when all populations produce mainly diapausing eggs. Bullhead Pond copepods also were reared at a fourth daylength, 11:40 corresponding to 10 March shortly before they switch from subitaneous to diapausing eggs.

The copepods were allowed to mature in the 2 L jars. Females carrying their first clutches were isolated individually in 15 ml of medium in 6-well tissue culture plates, and observed until either their eggs had hatched or they had dropped their egg sacs. The females were then placed in new 2 L jars with males and allowed to produce a second clutch. Copepods carrying their second egg clutches were reisolated in the tissue culture wells and egg hatching was again monitored. The process was continued until all females had produced three clutches. Females were pooled in 2 L jars because mating success was substantially higher in this arrangement than when males and females were isolated in small volumes of water. The procedure, however, does not permit a determination of the sequence of clutches made by individual females. Subitaneous and diapausing eggs cannot be distinguished under light microscopy, but we have shown previously (Hairston and Olds, 1984; Hairston and Munns, 1984) that in contrast to diapausing eggs, subitaneous eggs hatch rapidly, usually within one week after being laid. As in our earlier research, eggs that hatched within two weeks after production were designated subitaneous eggs, whereas those that had not hatched during this period were designated diapausing eggs. By these methods we established the fractions of subitaneous and diapausing eggs made by *D. sanguineus* from each of the three ponds when reared under the three or four experimental photoperiods. In addition, the fractions of egg types produced as first, second, and third clutches were determined independently in each treatment.

RESULTS

Populations of *Diaptomus sanguineus* from the three ponds have distinct photoperiodic responses (Fig. 2). In each case, copepods reared under short day lengths produced significantly greater fractions of subitaneous clutches than those reared under longer photoperiods (Ponds A and C, $\chi^2 = 116.2$ and 94.9 respectively, df = 2, $P \ll .001$; Bullhead Pond, $\chi^2 = 211.1$, df = 3, $P \ll .001$). Figure 2 illustrates both the results from the treatments described here, and those from a second experiment in which Bullhead Pond and Pond A copepods reared under winter conditions (8L:16D, 4 ± 1°C) produced only subitaneous eggs and those reared under summer conditions (16L:8D, 20 ± 1°C) made only diapausing eggs (Hairston and Olds, in prep.).

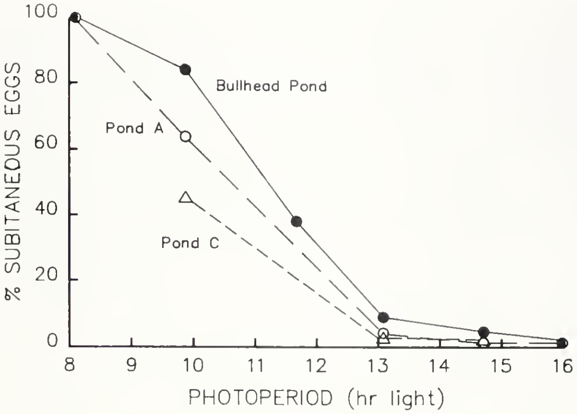

FIGURE 2. The fractions of subitaneous (immediately hatching) clutches of eggs produced by female *Diaptomus sanguineus* reared at the photoperiods illustrated. Data are for all clutches regardless of order of production. For sequence of clutch production see Table I. Experiments at intermediate day lengths were run at 9 ± 1°C, whereas those at 8L:16D and 16L:8D were run at 4 ± 1°C and 20 ± 1°C respectively.

The levels of response differed significantly among *D. sanguineus* drawn from different ponds. At day length 9:55 hours Bullhead Pond females made a greater fraction of subitaneous eggs than either Pond A or Pond C females ($\chi^2 = 14.67$ and 41.68 respectively, df = 1, $P < .001$), and Pond A females made a greater fraction of subitaneous eggs than Pond C females ($\chi^2 = 6.37$, df = 1, $P < .02$). At longer photoperiods where subitaneous egg production was generally low, Bullhead Pond results differed significantly from Pond C at day length 13:05 hours ($\chi^2 = 5.44$, df = 1, $P < .02$), and from Pond A at day length 14:45 hours ($\chi^2 = 3.91$, df = 1, $P < .05$).

Although others have found that diaptomid copepods tend to switch from making subitaneous eggs to diapausing eggs as they grow older (Roen, 1957; Champeau, 1970; Gehrs and Martin, 1974; Walton, 1985), and such a reproductive pattern is expected theoretically in highly variable environments (Hairston *et al.*, 1985; Hairston and Olds, in prep.), no clear trend of this sort is present in our results (Table I). Under a photoperiod of 9:55L both Pond A and Pond C females reduced the fraction of subitaneous eggs they produced in second compared to first clutches ($\chi^2 = 6.61$ and 7.89 respectively, df = 1, $P < .02$), but in each case they returned to making more subitaneous eggs in their third clutches than in their second clutches.

DISCUSSION

Our hypothesis was that the distinct reproductive phenologies of *Diaptomus sanguineus* living in three Rhode Island ponds (Fig. 1A) reflect unique adaptations to the seasonal events in those habitats. Specifically we proposed that copepods taken from different populations begin production of diapausing eggs at different times of year because they respond proximately to different photoperiods. In a broad sense the hypothesis is born out (Fig. 2), in that (1) the copepods make principally subitaneous eggs at short photoperiods and principally diapausing eggs at long photoperiods and (2) significant differences in this response pattern exist between populations. However, the change from subitaneous (nondiapausing) eggs to diapausing eggs occurs more gradually and over a much wider range of photoperiods than is consistent with the field data (*cf.*, Fig. 2 and Fig. 1B). Furthermore, the specific photoperiods at which

TABLE I

Fractions of subitaneous (immediately hatching) clutches of eggs as opposed to diapausing clutches, produced by female Diaptomus sanguineus *reared at four photoperiods, and the change in this fraction as the females aged (i.e. 1st, 2nd, and 3rd clutches)*

Photoperiod	Clutch sequence	Pond		
		Bullhead	A	C
9:55L–14:05D	1st	0.85 (72)[1]	0.62 (69)	0.53 (53)
	2nd	0.82 (50)	0.42 (24)	0.22 (32)
	3rd	0.90 (20)	0.50 (4)	0.78 (9)
11:40L–12:20D	1st	0.38 (131)		
	2nd	0.36 (75)		
	3rd	0.50 (2)		
13:05L–10:55D	1st	0.14 (98)	0 (55)	0.03 (72)
	2nd	0 (47)	0 (43)	0 (41)
	3rd	0 (6)	0.21 (24)	0.10 (10)
14:45L–9:15D	1st	0.04 (48)	0 (70)	0.02 (58)
	2nd	0 (9)	0 (28)	0.03 (32)
	3rd	— (0)	0 (12)	0 (5)

[1] The values in parentheses give the total numbers of reproducing females in each treatment.

the switches take place in the field populations differ markedly from those recorded in the laboratory.

Female copepods collected from Bullhead Pond carry principally subitaneous eggs until mid-March when day length exceeds 11.5 hours. The switch in egg types then occurs rapidly and by the end of March, at day lengths of 12.5 hours, nearly all have switched to making diapausing eggs (Fig. 1). In contrast, Bullhead Pond copepods cultured in the laboratory make a small, but significant, fraction of diapausing eggs when exposed to only about 10 hours of light (comparable to the day length on 25 January), and yet continue to make a significant fraction of subitaneous eggs at a photoperiod greater than 13 hours of light (comparable to the day length on 10 April). Half of the females in the field have switched to carrying diapausing eggs at a day length of 12.2 hours (or 24 March), whereas in the laboratory this point is reached much earlier at exposure to about 11.2 hours of light (comparable to 27 February). For Ponds A and C, the differences between the populations in nature and their behavior in the laboratory are even more striking. One half of the female copepods in Pond A have switched to making diapausing eggs when day length has reached about 14.3 hours (14 May), but in the laboratory 50% diapause lies at 10.6 hours of light (comparable to 12 March). In Pond C, one half of the females have switched to diapause at a day length of 13.9 hours (29 April), whereas in the laboratory 50% diapause occurs at less than 9.9 hours of day light (comparable to 25 January).

Not only do laboratory-reared *D. sanguineus* exhibit photoperiod responses inconsistent in timing with the patterns of reproduction recorded in the field, but the order of the responses is also incongruous (*cf.* Fig. 1B and Fig. 2). In the natural populations, Bullhead Pond females are the first to switch to producing diapausing eggs, followed by Pond C females, and finally by those in Pond A. In the laboratory, however, Pond C copepods switch to diapause at the shortest day lengths followed by Pond A and Bullhead Pond copepods. The fact that the differences in laboratory photoperiod responses between the three populations are statistically significant (Results) indicates that the dissimilarities are inherent. Since all the copepods were cultured under identical laboratory conditions, environmental sources of variation were presumably removed, and the populations from the three ponds apparently differ genetically.

It is clear that the natural populations of *D. sanguineus* must use environmental cues other than critical photoperiod to set the timing of diapause. The behavior observed in the laboratory, although real, represents only a single component of a more complex response pattern. Without further experimentation it is difficult to say what factors might be involved in a complete characterization of the environmental cue for the onset of diapause. The photoperiodic response of many animals is modifiable to some extent by temperature (*e.g.*, Beck, 1980), including the marine calanoid copepod *Labidocera aestiva* studied by Marcus (1982). As spring progresses into summer, *D. sanguineus* experiences both an increase in day length and an increase in water temperature, and one might suppose that each population is adapted to respond to a unique combination of these two environmental variables. Two difficulties with such a suggestion are (1) that exposing Pond A and C animals to a temperature abnormally low for the photoperiod used should have enhanced the production of subitaneous over diapausing eggs, but instead had the opposite effect, and (2) that even the appropriate temperature and photoperiod combination did not produce the correct response by Bullhead Pond copepods. A third type of environmental information available to the copepods at all three ponds is the direction and rate of change of photoperiod. Tauber and Tauber (1970) have shown for a lacewing (Neuroptera) that autumnal diapause can be induced at relatively long photoperiods if the insects are exposed to decreasing rather than constant day lengths. Sensitivity to such a change in day length, were it to exist in *D. sanguineus*, could explain both the rapidity and the timing of the onset of diapause. Whatever the environmental cue or cues used by the copepods to time the switch to diapause, discovering their precise nature will permit more detailed investigation of the evolutionary bases of interpopulation differences in reproductive phenology.

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EFFECTS OF ENRICHMENT ON REPRODUCTION IN THE OPPORTUNISTIC POLYCHAETE *STREBLOSPIO BENEDICTI* (WEBSTER): A MESOCOSM STUDY

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ABSTRACT

The influence of organic enrichment on growth and planktotrophic development of the spionid polychaete *Streblospio benedicti* Webster was examined in two mesocosm experiments conducted at the MERL facility, University of Rhode Island. Specimens of *S. benedicti* were collected and their reproductive traits monitored near the conclusion of a two-year eutrophication experiment, and in the middle of a sludge addition experiment. Nutrient (N, P, and Si) enrichments at 8× and 32× the average aerial input into Narragansett Bay, Rhode Island, resulted in increases in body length, segment number, and length per segment, and a doubling of brood size in *S. benedicti* females. These increases were substantially higher during May (12°C) than August (20°C). Enrichment effects were stronger in the 8× than 32× nutrient treatment. In the sewage sludge experiment body size increased 20% over control values at the highest (8×) sludge treatment level (nitrogen loading equivalent to the 8× nutrient treatment) but no significant increase was noted at the 4× sludge level, which received half as much nitrogen as the 8× sludge treatment. Mean brood size increased by a factor of 4.6 over controls in the 8× sludge treatment and by a factor of 2.3 in the 4× sludge treatment. Within the range of adult body sizes observed, brood size enhancement occurred independent of increased length or segment number in both nutrient and sludge enrichment treatments. The ability to translate elevated food supply directly into increased reproductive output may underly opportunistic dynamics in macrobenthos. Brood size enhancement of the magnitude observed probably contributes to the high *S. benedicti* densities found in polluted or organically enriched settings.

INTRODUCTION

Increased concern over pollution of harbors and bays during the 1960's and 1970's spawned interest in community structure and succession of estuarine macrofauna (reviewed in Pearson and Rosenberg, 1978). Many estuarine studies revealed life history characteristics and population dynamics in polychaetes, crustaceans, and bivalves which are defined as opportunistic (*e.g.*, Grassle and Grassle, 1974; McCall, 1975). Life history traits associated with opportunism in macrobenthos include small size, rapid colonization ability, short generation time, high reproductive rate (r), and high mortality rate (Grassle and Grassle, 1974). Brood protection is also a common feature among many opportunistic polychaetes, including *Capitella* spp., *Polydora ligni*, and *Streblospio benedicti* (Grassle and Grassle, 1974; Levin, 1984a), and among peracarid crustaceans (Barnes, 1980). However, not all opportunists brood young; the bivalve *Mulinia lateralis* has completely planktonic development (Chanley and Andrews, 1971).

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Opportunistic species colonize and dominate benthic assemblages during or following bottom disturbances, such as those caused by release of sewage or industrial wastes (Reish, 1971; Boesch, 1973), dredging and spoil disposal (Oliver, 1979), nutrient additions and subsequent eutrophication (Nixon *et al.*, 1984), oil spills (Grassle and Grassle, 1974; Sanders *et al.*, 1980), or severe storms and hurricanes (Boesch *et al.*, 1976; Rhoads and Boyer, 1983). Biological disturbance of sediment, such as that caused by digging activities of crabs, bottom fish, rays, or whales may also lead to colonization and persistence of opportunistic taxa (Virnstein, 1977; Van Blaricom, 1982; Levin, 1984a, Oliver and Slattery, 1985).

Pearson and Rosenberg (1978) make a distinction between disturbance and enrichment opportunists. They cite *Capitella* spp., *Streblospio benedicti, Scolelepis fuliginosa,* and dorvilleids as examples of species which colonize disturbances specifically resulting from organic enrichment. Species in the genus *Streblospio,* common in North America and Europe, are often numerically dominant in polluted or enriched estuarine habitats. *S. benedicti* recruits are typically found in caged settlement containers which trap fine organic particulates. Densities of over 100,000 individuals/m^2 have been reported in settling containers and clearings exposed for 2 weeks (McCall, 1975; Virnstein, 1977; Levin, 1984a). Enhanced recruitment may have resulted from either active habitat selection or passive entrainment (Hannan, 1984).

Streblospio is not limited to enriched or disturbed habitats. *S. benedicti* has been reported from most of the shallow estuaries, marshes, and mudflats studied in the United States, with the exception of some in Washington and Hawaii (Levin, 1984b). This species is often a moderate component of the background community but population size can grow exponentially in response to disturbance. Similar behavior is documented for *Capitella* spp. and *Polydora ligni* (Pearson and Rosenberg, 1978).

The mechanisms underlying opportunistic responses are just now being investigated. High population growth rates are thought to derive from life history characteristics while high mortality rates have been attributed to competition from later stages (Grassle and Grassle, 1974; Pearson and Rosenberg, 1978; Gallagher *et al.*, 1983; Gallagher, pers. comm.). The dynamics of *Capitella* sp. I fed Gerber's cereal, was studied in the laboratory by Chesney and Tenore (1985a, b). They observed high amplitude population fluctuations in the presence and absence of induced mortality (artificial predation) over 90 weeks. High mortality occurred and they concluded that *Capitella* sp. I was unlikely to achieve equilibrium dynamics on its own. Based on these observations Chesney (1985) suggests that inherent life history traits (*e.g.*, reproductive lags and a tendency to overshoot carrying capacity) rather than competition are probably responsible for population declines of opportunists observed during succession in soft sediments. However, the meiofaunal community present in these experiments (Alongi, 1985) was not considered in the interpretation of *Capitella* sp. I dynamics and may have influenced resource availability.

The ability to rapidly translate increases in food availability to increased reproductive output should lead to opportunistic population dynamics, particularly if offspring remain to further utilize the enhanced food supply. Production of more gametes and offspring, independent or instead of concomitant somatic growth (which uses energy and takes time), should also enhance rapid population growth in opportunists. Eckelbarger (in press) suggests that opportunism among polychaetes is limited to species with brief gametogenic cycles, which permit numerical response to specific conditions before those conditions disappear. Spionid and capitellid polychaetes can produce mature gametes only a few weeks after initiating gametogenesis (Eckelbarger, in press).

This paper examines the reproductive responses by *Streblospio benedicti* to different forms of organic enrichment. *S. benedicti* was studied in two experiments conducted in mesocosms at the Marine Ecosystems Research Laboratory (MERL), University of Rhode Island. One was a eutrophication experiment involving nutrient additions, the other involved addition of secondary sewage sludge. In an earlier paper (Levin, 1984b) I suggested that the widespread success of *S. benedicti* may be attributable to the occurrence of both planktotrophic and lecithotrophic modes of development in this species. The research presented here considers only *S. benedicti* with planktotrophic development and examines the plasticity of reproductive traits, such as egg and brood sizes.

Streblospio benedicti *background*

Streblospio benedicti Webster (Spionidae) inhabits the upper 2–3 cm of muddy sediments and constructs ephemeral tubes of fine-grain particles. This species is both a suspension feeder and surface-deposit feeder on plankton, organic aggregates, and sediments. Rod-shaped fecal pellets are deposited outside the tube (Levin, 1981). *S. benedicti* exhibits both planktotrophic and lecithotrophic modes of development in North America, but only planktotrophic development has been reported for *S. benedicti* in Narragansett Bay and in the MERL tanks (Levin, 1984b., J. P. Grassle, pers. comm.). Oogenesis takes place in paired ovaries in anterior segments (Eckelbarger, 1980) and fertilized ova enter paired dorsal brood pouches where development proceeds. Larvae are brooded to a 3–5 setiger stage, approximately 220 μm in length. Following release from brood pouches larvae usually feed in the plankton for 10–21 days before settlement (Levin, 1984b). Females can release hundreds of planktotrophic larvae per brood (Levin, 1984b) and may produce as many as 14 broods in a lifetime (Levin, DePatra, and Creed, in prep.).

MATERIALS AND METHODS

Streblospio benedicti was sampled from the MERL mesocosms, located at the University of Rhode Island on the lower West Passage of Narragansett Bay, RI. Sampling was conducted on 12 May and 17 August 1983, towards the conclusion of a 2-year eutrophication experiment and on 26 July 1984 during a 3-month sewage sludge enrichment experiment. The design of the MERL mesocosms is described in Pilson *et al.* (1979). System-wide results of the first year of the eutrophication experiment are presented in Nixon *et al.* (1984) and preliminary results of the sludge experiment are described in Oviatt (1984). Methodology critical to this investigation will be reviewed here.

The MERL mesocosms were 5.5 m high cylindrical tanks (1.83 m diameter). The tanks received seawater from Narragansett Bay at a rate sufficient to completely replace the water every 27 days. The mesocosms were mixed four times a day with rotating plungers on a schedule designed to mimic tidal currents and to resuspend bottom sediments to similar levels as in the Bay. The walls of the tanks were brushed twice a week to prevent fouling. Each tank contained a tray 2.52 m^2 × 40 cm deep filled with sediments. These sediments were collected intact from Narragansett Bay (using a 0.25 m^2 box core) between 28 April and 8 May 1981 for the eutrophication experiment. Fresh sediments were collected from central Narragansett Bay in October 1983, prior to the start of the sludge experiment. On 7 June 1984, surface sediments (to 2 cm depth) were removed from all tanks to eliminate a bloom of the tunicate *Mogula manhattensis* and fresh sediments from the Bay were added. Narragansett Bay sediments were muddy, containing roughly 83% silt-clay at the collection site (Hunt and Smith, 1983). The dominant taxa in these sediments at the start of the eutrophication experiment were the polychaetes *Mediomastus ambiseta, Polydora ligni, Streblospio benedicti,* and *Chaetozone* sp., and the bivalves *Nucula annulata* and *Yoldia limatula* (Grassle *et al.,* 1985).

Eutrophication experiment

The eutrophication experiment involved daily additions of inorganic nitrogen, phosphorus, and silicon (Molar ratio 12.80:1.00:0.91) at 1×, 2×, 4×, 8×, 16×, and 32× the average areal input (of sewage and runoff) to Narragansett Bay (Table I). Each of six tanks received a different enrichment level and three tanks remained as unenriched controls. Nutrient additions began on 1 June 1981 and continued daily through 26 September 1983. Specimens of *Streblospio benedicti* were collected from 2 control tanks (Nos. 5 and 8), the 8× enrichment tank (No. 1), and the 32× enrichment tank (No. 7) on 12 May and 17 August 1983, approximately 2 and $2\frac{1}{4}$ years from the start of the experiment. The *S. benedicti* adults collected must have entered the tank as larvae from Narragansett Bay or were produced by adults established within the tank sediments. They could not have been individuals collected directly from the bay at the start of the experiment, since the lifespan of *S. benedicti* is <12 months (Levin, DePatra, and Creed, in prep.). Average temperature in the MERL mesocosm tanks was 12–13°C in May 1983 and 20–21°C in August 1983. Because this large temperature difference could have significant effects on reproduction, I consider the two sampling dates to represent two temperature treatments rather than replicate samplings.

Streblospio benedicti was collected from the upper 4 cm of MERL tank sediments using 1″ diameter cylindrical cores (5.067 cm^2, n = 5 cores/tank on each sampling date). Sediments were sieved through a 500-μm screen and all *S. benedicti* were sorted live under a dissecting microscope. Stage of maturity and sex were noted for all specimens. Females brooding young were isolated in petri dishes, anesthetized in 5% $MgCl_2$, and the following reproductive traits were measured: length (mm), number of setigers, position of the first gametogenic setiger, number of ova/ovary, ovum diameter (μm), number of brood pouches, number of larvae/brood pouch, and number of larvae/brood.

TABLE I

Experimental enrichments in the MERL eutrophication and sewage sludge experiments

Eutrophication experiment 1 June 1981–26 September 1983

	Daily additions (millimoles/m^2)		
Treatment level	N	P	Si
0x	–	–	–
8x	23.04	1.798	1.643
32x	92.03	7.19	6.570

Sewage sludge experiment 18 June 1984–21 September 1984

	Daily additions (millimoles/m^2)			
Treatment level	C	N	P	Si
0x	–	–	–	–
4x sludge	105	14.5	2.07	0.38
8x sludge	210	29.0	4.13	0.77

Sewage sludge experiment

The sewage sludge experiment consisted of seven treatments. Nutrients (N, P, and Si) were added daily to 3 tanks at 1×, 4×, and 8× enrichment levels, as in the eutrophication experiment. Primary and secondary sewage sludge from the Cranston, Rhode Island, Water Pollution Control Facility was added daily at 1×, 4×, and 8× levels to generate 3 treatments which received total nitrogen at loading rates equivalent to the nutrient treatments. Three unenriched tanks served as controls. The daily enrichments began on 12 June 1984 and were terminated on 22 September 1984. *Streblospio benedicti* was sampled in 2″ diameter cores ($17.57 \text{ cm}^2 \times 4$ cm deep) from the 4× and 8× nutrient tanks, the 4× and 8× sludge tanks, and 3 control tanks on 26 July 1984. Five to eleven cores were collected from each tank though time did not permit processing of all cores. Sediments were sieved and *S. benedicti* was sorted and measured as described for the eutrophication experiment. J. P. Grassle provided density data for *S. benedicti* collected the preceding day using 1″ diameter cores ($5.067 \text{ cm}^2 \times 4$ cm deep) and a 300-μm screen (Grassle and Grassle, 1984; Grassle *et al.*, 1985). Few reproductive *S. benedicti* were found in the 1984 nutrient enrichment tanks, so only the sludge enrichment and control treatments are considered here.

Statistical analyses

Statistical tests were carried out with SAS software (Ray, 1982). All analyses except for regressions of brood size on adult length and setiger number were performed on untransformed data. One-way ANOVAs were used to analyze effects of enrichment level on *S. benedicti* densities in the eutrophication and sludge experiments. A two-way ANOVA and a SNK *a posteriori* test were performed to evaluate effects of enrichment level and month on female length, segment number, the first gametogenic setiger, ovum diameter, and numbers of ova/ovary, paired brood pouches, larvae/brood pouch, and larvae/brood in the eutrophication experiment. Effects of sludge level on these same parameters were analyzed using a one-way ANOVA and SNK test. A comparison was made of all 9 enrichment treatments across experiments, (May 1983, August 1983, and July 1984) using a one-way ANOVA and a SNK test for each reproductive character. Data from the eutrophication and sludge experiments were combined to determine Pearson product-moment correlations between reproductive traits.

Brood size data from the eutrophication and sludge experiments were log transformed to satisfy least-squares assumptions prior to performing regressions of brood size on adult length and on setiger number. Homogeneity of variances was examined using F tests to compare error mean squares from regressions for individual treatments. No departures from homogeneity were observed within each experiment. Regressions of log brood size on both adult length and on setiger number were performed across enrichment levels (separately in the eutrophication and sludge experiments), allowing for different intercepts and slopes.

RESULTS

Eutrophication experiment

Streblospio benedicti densities in each enrichment treatment are shown in Table II for samples collected in May and August 1983. In May *S. benedicti* exhibited no density difference among enrichment levels ($F_{3,16} = 1.92$, $P > 0.05$). Mean densities in all treatments ranged from 2.8 to 9.6 individuals/5.07 cm^2 core (5,600 to 19,200

Table II

Density of Streblospio benedicti *in the MERL eutrophication experiment*

Food level	$\bar{x}$ Density (#/core)	S.D.	n (# of cores)	Core size (cm^2)	$\bar{x}$ Density ($\#/m^2$)	% Females brooding
12 May 1983						
0x (Tank 5)	9.60	6.20	5	5.07	19,200	80
0x (Tank 8)	2.80	1.48	5	5.07	5,600	55
8x (Tank 1)	4.00	4.38	5	5.07	8,000	75
32x (Tank 7)	4.80	4.70	5	5.07	9,600	58
17 August 1985						
0x (Tank 5)	4.60	2.94	5	5.07	9,200	50
0x (Tank 8)	0.00	0.00	4	5.07	0,000	—
8x (Tank 1)	25.60	16.33	5	5.07	51,200	50
32x (Tank 7)	31.00	11.64	4	5.07	62,000	58

(Mesh size = 500 μm).
One-way ANOVA on density.
May: $F_{3,16} = 1.92$, $P > 0.05$.
August: $F_{3,14} = 7.81$, $P < 0.005$.

individuals/m^2). Three months later, in August, the 8× and 32× enrichment tanks exhibited mean densities equivalent to 25.6 and 31.0 individuals/core respectively (51,200 and 62,000 individuals/m^2). These values were significantly higher than control values in August, but not different from each other ($F_{3,14} = 7.81$, $P < 0.005$). On both sampling dates at least 50% of the females collected in each treatment were brooding larvae (Table II).

The two control (0×) tanks, sampled in May, exhibited between-tank differences in number of ova/ovary ($\bar{x} = 6.2$ in tank 8 *vs.* $\bar{x} = 4.2$ in tank 5, one-way ANOVA, $P = 0.007$). However, there was no significant difference in brood size. May reproductive data from the two control tanks were pooled in analyses of enrichment level effects. Mean values of female reproductive traits are given for *S. benedicti* at 0×, 8×, and 32× enrichments in May and at 8× and 32× enrichments in August in Table III. Unfortunately, only two reproductive females were found in the control (0×) tanks in August, so analysis of food level effects for August does not include the 0× treatment.

During May, female body length was significantly greater in the 8× and 32× enrichment tanks than in the controls ($P = 0.0087$), but in both May and August, body length did not differ between the 8× and 32× treatments. The number of setigers was significantly greater in 8× than control treatments during May ($P = 0.0304$), but no difference in setiger number was observed between the 32× treatment and controls in May or between 8× and 32× treatments in both May and August. Between-month comparisons of body size, for 8× and 32× treatments, suggest that in August there was a decrease in body length ($P = 0.0090$) but no accompanying change in segment number (Table III, Fig. 1). Length per segment was greater in May than August in enriched tanks. Females sampled in August may have been younger, summer recruits, while the larger individuals sampled in May probably overwintered in the tanks.

As one trait responds to treatments other traits may be constrained to follow, thus correlations among reproductive characters must be considered in interpretation of treatment effects. Measures of body size (length and setiger number) were positively correlated with numbers of ova per ovary (r = .55 for length, r = .44 for setiger number), total number of paired brood pouches (r = .73, r = .82), number of larvae per brood pouch (r = .52, r = .36), and number of larvae per brood (r = .69, r = .61)

TABLE III

Reproductive traits of Streblospio benedicti *in the MERL eutrophication experiment*

	12 May 1983			17 August 1985		2-way ANOVA	
	0x	8x	32x	8x	32x		
	x̄ (S.D.)	x̄ (S.D.)	x̄ (S.D.)	x̄ (S.D.)	x̄ (S.D.)	Food	Month
Adult length (mm)	5.2 (1.4) n = 36	7.2 (1.9) n = 11	6.1 (1.6) n = 18	5.7 (1.2) n = 17	4.8 (1.0) n = 19	$P = .0087$	$P = .0090$
	b	a					
No. of setigers	42.6 (6.0) n = 35	49.4 (6.1) n = 12	46.2 (5.8) n = 18	45.4 (5.8) n = 17	41.6 (4.4) n = 19	$P = .0304$	NS
		a					
	b		b				
First gametogenic setiger	10.1 (0.6) n = 36	9.8 (0.8) n = 13	10.0 (0.6) n = 18	10.6 (1.1) n = 16	10.6 (0.8) n = 15	NS	NS
Ovum diameter (μm)	56.1 (23.2) n = 38	60.0 (21.7) n = 13	61.3 (27.3) n = 19	50.8 (18.3) n = 12	56.7 (19.5) n = 15	NS	NS
No. of ova/ovary	5.8 (1.9) n = 37	8.1 (2.8) n = 9	5.9 (1.9) n = 15	6.1 (1.9) n = 11	4.1 (1.0) n = 13	$P = .0002$	$P = .0011$
	b	a	b	a	b		
No. of paired brood pouches	9.0 (2.8) n = 37	14.0 (3.6) n = 13	11.8 (2.6) n = 17	9.2 (2.7) n = 17	9.5 (2.2) n = 17	NS	$P = .0228$*
	b	a		b	a		
No. of larvae/brood pouch	6.8 (2.3) n = 18	10.4 (3.4) n = 11	7.4 (1.7) n = 14	7.4 (1.6) n = 10	5.0 (1.4) n = 2	$P = .0011$	$P = .0021$
	b	a	b				
No. of larvae/brood	103.9 (54.7) n = 28	278.2 (143.3) n = 13	184.3 (90.1) n = 15	131.6 (45.6) n = 14	67.6 (41.7) n = 21	$P = .0001$	$P = .0002$
		a		a	b		
	b		b				

* Significant food-month interaction $P = .0177$.

(Lines show differences among means analyzed for each month separately.)

NS = not significant.

(Table IV). These correlations suggest that changes in body size may also lead to changes in fecundity. Numbers of ova and larvae were also highly correlated with one another (r = .61), thus we might expect to see them respond in identical fashion to food and temperature (month) treatments. Body size was not correlated with egg size or with position of the first gametogenic setiger (Table IV).

Both enrichment level and month (temperature) had significant effects on ovum number ($P = 0.0002$ for food, $P = 0.0011$ for month), numbers of larvae per brood pouch ($P = 0.0011$, $P = 0.0021$) and brood size ($P = 0.0001$, $P = 0.0002$) (Table III). Ovum size and position of the 1st gametogenic setiger were unaffected (Table III). During May the mean number of larvae produced per brood was 167% higher than

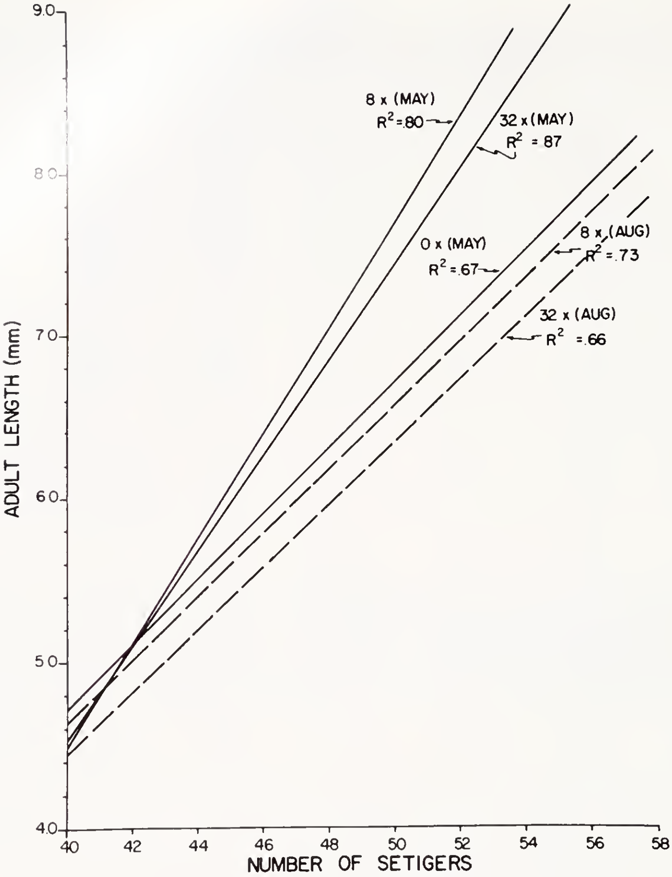

FIGURE 1. Least-square regression of *Streblospio benedicti* segment number on length in the MERL eutrophication experiment.——May 1983; ----- August 1983.

May 0× (control): $y = -3.11 + 0.196x$ $P < .0001$

8× : $y = -6.59 + 0.277x$ $P < .0001$

32× : $y = -5.65 + 0.255x$ $P < .0001$

August 8× : $y = 2.65 + 0.183x$ $P < .0001$

32× : $y = 2.99 + 0.187x$ $P < .0001$

Slopes for May 8× and 32× treatments differ significantly from the others.

the controls ($\bar{x}$ = 104) in the 8× enrichment ($\bar{x}$ = 278) and 77% higher than the controls in the 32× enrichment ($\bar{x}$ = 184). However, only the 8× and 0× treatments differed significantly from one another ($P = 0.0001$). Seven females produced broods > 300 larvae (the largest was 548); all were large individuals from enriched treatments.

Brood size was highly correlated with adult length ($r = .69$, $P < 0.0001$) and segment number ($r = .61$, $P < 0.0001$). Scatter plots of May brood sizes as a function of length and setiger number are given for each enrichment level in Figures 2a and b. Although correlation does not necessarily indicate a causal relationship, the well known

TABLE IV

Correlation coefficients of Streblospio benedicti *reproductive traits in the MERL eutrophication and sludge experiments combined*

	Adult length	No. of setigers	First gametogenic setiger	Ovum diameter	No. of ova/ovary	No. of paired brood pouches	No. of larvae/brood pouch	No. of larvae/brood
Adult length	1.0	.86	NS	NS	.55	.73	.52	.69
No. of setigers		1.0	NS	NS	.44	.82	.36	.61
First gametogenic setiger			1.0	NS	−.20	NS	NS	−.21
Ovum diameter				1.0	.21	NS	NS	NS
No. of ova/ovary					1.0	.40	.54	.61
No. of paired brood pouches						1.0	.50	.70
No. of larvae/brood pouch							1.0	.71
No. of larvae/brood								1.0

All values are significant at $P < .05$.
NS = not significant.

association between body size and fecundity raises the possibility that the increases in brood size observed in May enrichment treatments (8× and 32×) resulted solely from increases in body size. To examine this possibility, log brood size was regressed on both adult length and on setiger number, across enrichment levels. Both regressions yielded r^2 values > 0.995. Tests for differences among slopes across enrichment levels were significant ($P < 0.0001$) for regressions of log brood size on setiger number ($F_{3,46} = 24.5$) and length ($F_{3,47} = 23.5$). However, fitted lines for the three enrichment levels did not intersect within the range of observed setiger numbers (8× values > 32× > 0×). The 0× and 32× treatment lines crossed only at the very largest adult lengths observed. Predicted brood sizes for the 8× treatment were greater than those for the 0× and 32× treatments within the entire range of body sizes observed. Thus, though the regression lines for each enrichment level were not parallel, their intersection took place at biologically meaningless (unrealistically large) body and brood sizes.

A between-month comparison shows significantly ($P = 0.0002$) smaller broods were produced in August (Table III, Fig. 3). Brood sizes were less than half those observed in May; mean brood size was 132 for the 8× treatment and 68 for the 32× treatment.

Sewage sludge experiment

The mean density of *S. benedicti* was considerably lower in the control (0× enrichment) tanks [$\bar{x} = 4.63$ individuals/17 cm^2 core (2,635/m^2)] than in the 4× sludge treatment [$\bar{x} = 60$ individuals/core (34,140/m^2)] or the 8× sludge treatment [$\bar{x} = 22$ individuals/core (12,515/m^2)] ($F_{4,10} = 17.01$, $P < 0.001$) on 26 July 1984, one month into the sewage experiment (Table V). Density estimates based on two 1″ cores per tank provided by J. P. Grassle were 4–5 times higher than those from the 2″ diameter cores (due to use of a finer mesh for processing sediment), but yielded consistent differences among treatments (Table V). One control tank (No. 3) produced all except one of the control females brooding young. Fifty-five percent of females were brooding in that control tank, 88% were brooding in the 4× sludge treatment, and 85% were brooding in the 8× sludge treatment (Table V).

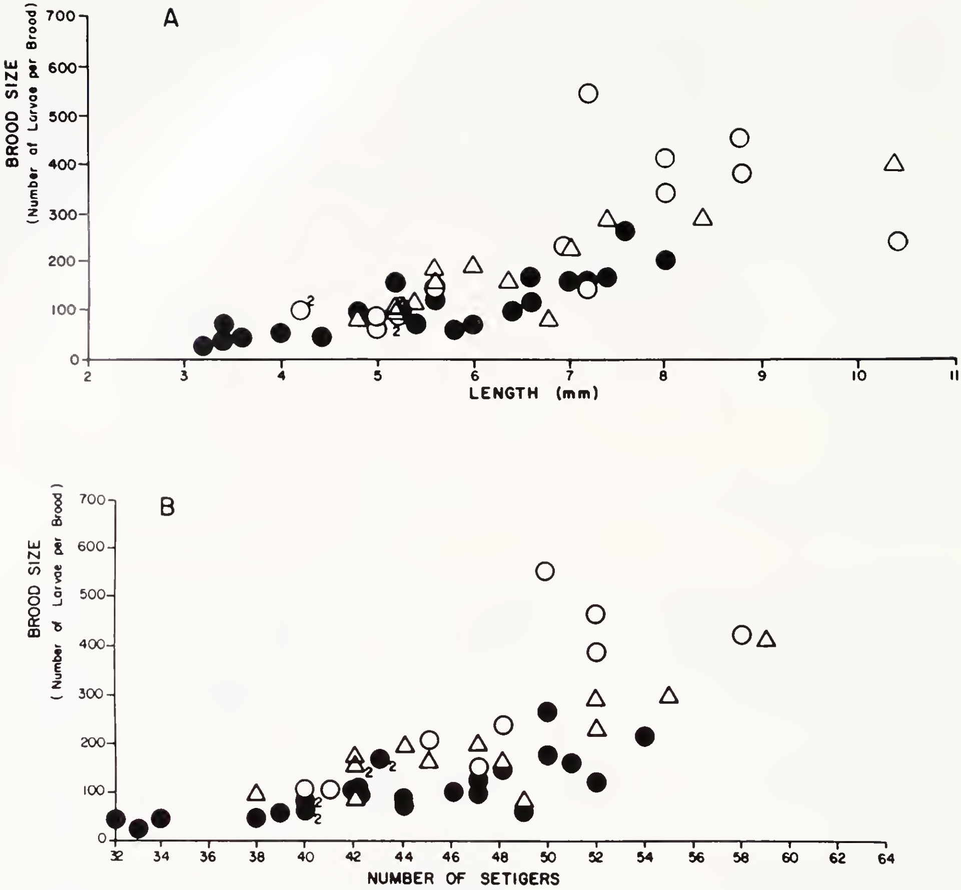

FIGURE 2. *Streblospio benedicti* brood size as a function of: A) length and B) setiger number in the MERL eutrophication experiment, 12 May 1983. ● = controls (Tanks 5 and 8); ○ = 8× enrichment (Tank 1); △ = 32× enrichment (Tank 7).

Evaluation of enrichment level effects on female traits indicate that female length ($P = 0.0319$) and number of setigers ($P = 0.0244$) were significantly greater in the 8× sludge treatment than in the 4× and 0× treatments, which did not differ from one another (Table VI). Females in the 8× sludge treatment also produced more ova/ovary ($P = 0.0002$), more larvae per brood pouch ($P = 0.0005$), and more larvae per brood ($P = 0.0001$) than the other two treatments, which did not differ significantly from one another (Table VI). Mean brood size in the 8× sludge treatment ($\bar{x} = 462$) was almost twice that of the 4× sludge tank ($\bar{x} = 235$) and over 4 times that of the controls ($\bar{x} = 100$). The only trait for which the 4× sludge treatment differed from the control was in the total number of paired brood pouches ($P = 0.0004$). As in the eutrophication experiment, position of the first gametogenic segment and ovum size were not affected by sludge level.

A scatter plot of brood size as a function of length (Fig. 4a) and segment number (Fig. 4b) for each sludge enrichment level reveals an exponential increase in brood size with body size. Five individuals in the 8× sludge treatment produced over 500

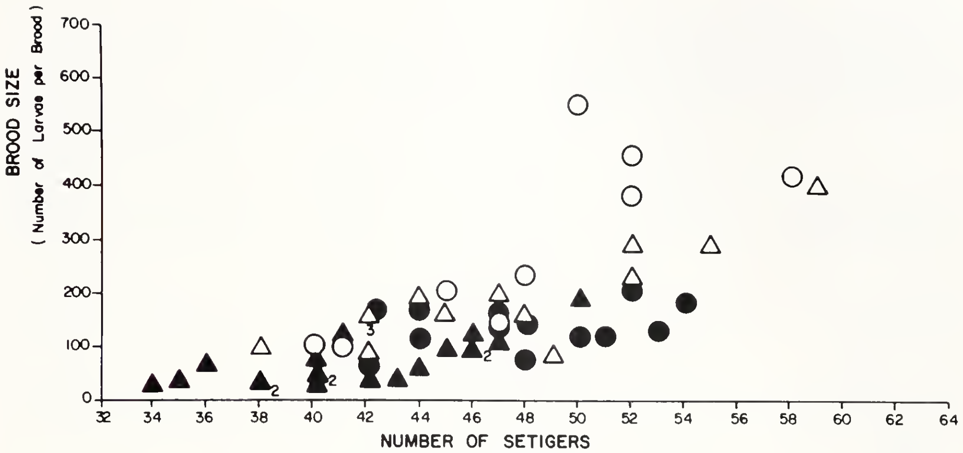

FIGURE 3. *Streblospio benedicti* brood size as a function of setiger number in the enriched treatments of the MERL eutrophication experiment. 12 May 1983: ○ = 8× enrichment, △ = 32× enrichment. 17 August 1983: ● = 8× enrichment, ▲ = 32× enrichment. The numbers indicate identical data points.

larvae/brood (Fig. 4). The maximum brood size observed, 1058, far surpassed any values reported for *S. benedicti* in a laboratory or field situation. Regressions of log brood size on adult length and on setiger number, across sludge enrichment levels, yielded r^2 values > 0.994. Tests for differences among slopes across sludge levels were significant for regressions of log brood size on setiger number ($F_{3,35} = 14.59$, $P < 0.0001$) and on length ($F_{3,36} = 20.50$, $P < 0.0001$). However, fitted lines for the three sludge levels did not intersect within the range of observed adult segment numbers or body lengths. For all sizes of worms capable of reproducing, predicted brood sizes were greater in the 8× sludge treatment than in the 4× sludge treatment, and both were greater than those in the 0× sludge treatment.

TABLE V

Density of Streblospio benedicti *in the MERL sewage sludge experiment (26 July 1984)*

Food level	x̄ Density (#/core)	S.D.	n (# of cores)	x̄ Density (#/m²)	% Females brooding
0x (Tank 3)	5.64 (4.0)	2.90	11 (2)	3,209 (7,905)	55
0x (Tank 5)	3.00 (4.0)	1.41	3 (2)	1,707 (7,905)	No mature females
0x (Tank 8)	1.50 (4.5)	1.50	2 (2)	854 (8,893)	100 (one mature female)
4x (Tank 6)	>60.00 (84.0)	—	1 (2)	>34,140 (165,019)	88
8x (Tank 2)	22.00 (25.0)	10.42	4 (2)	12,518 (49,407)	85

( )—indicates data from two cores/tank collected on 25 July 1984 by J. P. Grassle. Screen Size = 300 μm; Core Size = 5.07 cm².

(Screen Size = 500 μm, Core Size = 17.57 cm²); One-way ANOVA on density: $F_{4,10} = 17.01$, $P < 0.001$.

TABLE VI

Reproductive traits of S. benedicti *in the MERL sludge experiment 26 July 1984*

	Sludge enrichment level			One-way ANOVA	
	0x	4x	8x		
	x̄ (S.D.)	x̄ (S.D.)	x̄ (S.D.)		*P*
Adult length (mm)	5.3 (1.4) n = 11	5.8 (1.0) n = 19	6.8 (2.0) n = 14	$F_{2,41} = 3.75$	.0319
No. of setigers	41.4 (5.5) n = 11	45.4 (4.8) n = 18	47.9 (6.7) n = 14	$F_{2,40} = 4.08$	.0244
First gametogenic setiger	9.3 (0.7) n = 12	9.6 (0.5) n = 18	9.5 (0.9) n = 13	$F_{2,40} = 0.39$	NS
Ovum diameter (μm)	60.9 (25.0) n = 11	55.0 (23.6) n = 17	58.6 (23.8) n = 14	$F_{2,39} = 0.21$	NS
No. of ova/ovary	6.2 (1.3) n = 11	5.4 (1.7) n = 16	8.4 (3.0) n = 13	$F_{2,37} = 7.14$	.0002
No. of paired brood pouches	9.0 (2.6) n = 12	12.4 (3.3) n = 19	14.3 (3.3) n = 14	$F_{2,42} = 9.44$	.0004
No. of larvae/brood pouch	7.1 (1.7) n = 12	9.0 (1.4) n = 10	11.7 (3.7) n = 13	$F_{2,32} = 9.86$	.0005
No. of larvae/brood	100.0 (54.0) n = 11	235.1 (107.1) n = 19	462.8 (296.4) n = 13	$F_{2,40} = 12.78$	.0001

Lines show significant differences among means.
NS = not significant.

Comparison of the eutrophication and sewage sludge experiments

A comparison was made of all nine treatments (3 nutrient levels in May 1983, 2 nutrient levels in August 1983, and 3 sludge levels in July 1984) for each reproductive character (Table VII). May 1983 and July 1984 unenriched controls showed no significant difference in any trait except position of the first gametogenic setiger (Table VII). *S. benedicti* from the 8× nutrient addition in May 1983, were most similar to the 8× sludge addition in July 1984. These treatments produced adults which were significantly longer, had more segments, more brood pouches, and greater brood size than the May 1983 and July 1984 controls. The 4× sludge treatment and May 32× nutrient addition generally exhibited the next highest values for these traits, but did not always differ significantly from control values. Females produced significantly larger broods in the 8× sludge treatment than in any other treatment.

DISCUSSION

The sacrifice of replication for an increased number of treatments seems to be a common difficulty in the design of mesocosm and other large-scale experiments (see

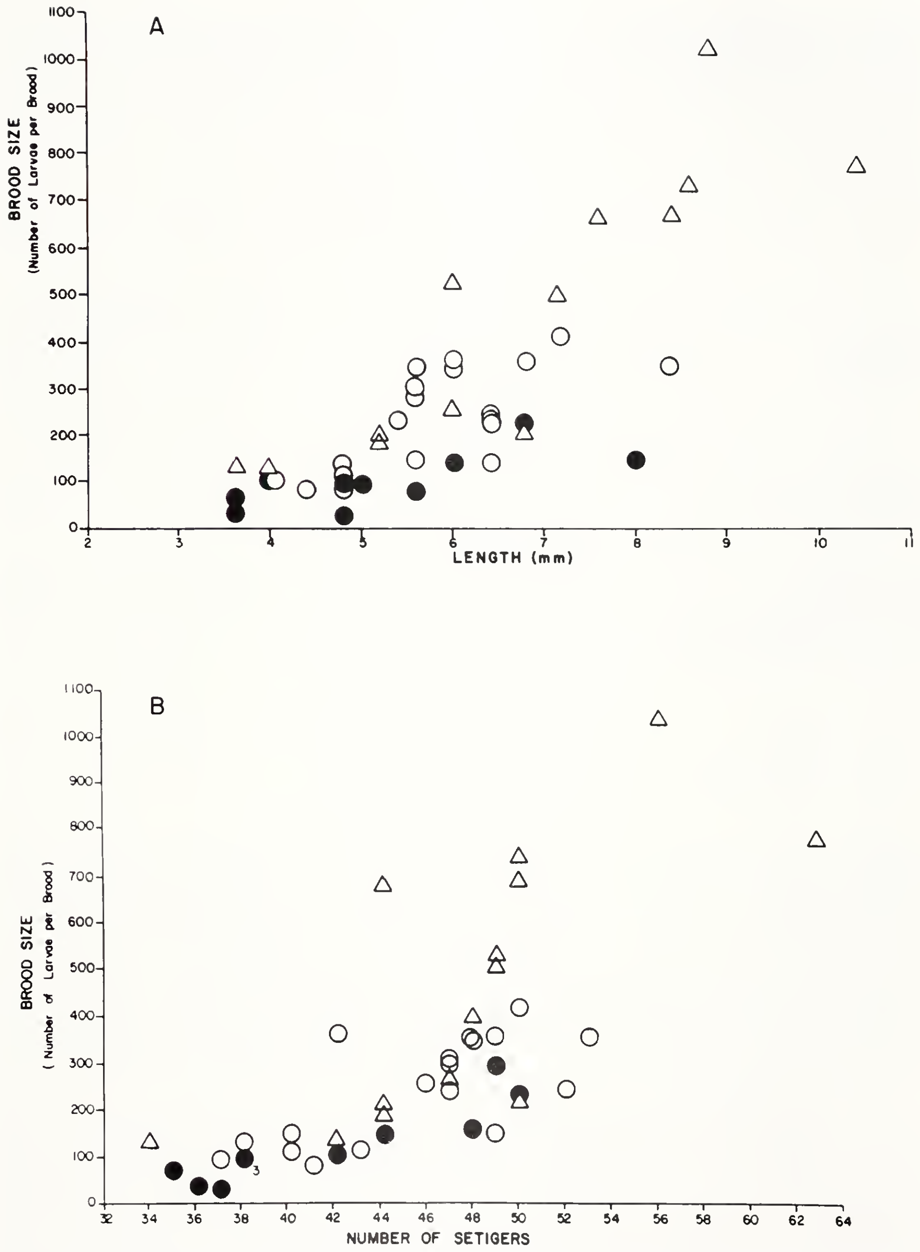

FIGURE 4. *Streblospio benedicti* brood size as a function of: A) length and B) setiger number in the MERL sewage sludge experiment. 26 July 1984. ● = 0× control (Tank 3); ○ = 4× sludge addition (Tank 6); △ = 8× sludge addition (Tank 2).

Hurlbert, 1984; Smith *et al.*, 1984 for discussion). The lack of tank replication of enrichment treatments in both the MERL nutrient and sewage experiments must temper the interpretation of the data. The fact that the control (0×) tanks behaved

TABLE VII

Comparison of Streblospio benedicti *reproductive traits among all MERL treatments*

1. = 0x Nutrients May '83
2. = 8x Nutrients May '83
3. = 32x Nutrients May '83
4. = 8x Nutrients August '83
5. = 32x Nutrients August '83
6. = 0x Sludge July '84
7. = 4x Sludge July '84
8. = 8x Sludge July '84

	One-way ANOVA	Student-Neumann-Keuls Test
Adult length	$F_{7,137} = 4.97, P = .0001$	2 8 3 7 4 6 1 5
No. of setigers	$F_{7,136} = 4.04, P = .0005$	2 8 3 7 4 1 5 6
First gametogenic setiger	$F_{7,133} = 6.39, P = .0001$	5 4 1 3 2 7 8 6
Ovum diameter (μm)	$F_{7,131} = 0.32, P = \text{NS}$	3 6 2 8 5 1 7 4
No. of ova/ovary	$F_{7,117} = 6.19, P = .0001$	8 2 4 6 3 1 7 5
No. of paired brood pouches	$F_{7,138} = 10.10, P = .0001$	8 2 7 3 5 4 1 6
No. of larvae/brood pouch	$F_{7,82} = 7.12, P = .0001$	8 2 7 3 4 6 1 5
No. of larvae/brood	$F_{7,126} = 17.61, P = .0001$	8 2 7 3 4 1 6 5

NS = not significant.

similarly lends little confidence that single tanks are an adequate reflection of all possible outcomes of particular levels of enrichment. Each treatment exhibited its own benthic dynamics throughout the experiments. Some tanks evolved oxygen stress in response to nutrient loadings or high densities of competitors or predators while others did not (J. P. Grassle, pers. comm.; Frithsen *et al.*, 1985). Within a treatment, macrofaunal community composition varied greatly from year to year (Frithsen *et al.*, 1985). However, several benthic species exhibited essentially linear responses to level of nutrient additions after the first summer of the eutrophication experiment (Grassle and Grassle, 1984; Grassle *et al.*, 1985).

To understand responses of *S. benedicti* it is necessary to consider both biotic and abiotic influences on reproduction. During the eutrophication experiment in both May and August 1983, the length, segment number, egg production, and brood size of *S. benedicti* in the 32× enrichment treatment fell below those of the 8× enrichment tank (Table III). Despite a four-fold increase in nutrient loading, it appears that *S. benedicti* did not respond in the 32× treatment. Intraspecific competition has been shown to influence somatic growth and reproductive allocation in benthic invertebrates (Peterson, 1982; Zajac, 1985). However, there was no detectable difference between *S. benedicti* densities in the 8× and 32× treatments throughout the spring and summer of 1983 (Table II, J. P. Grassle, pers. comm.; J. Frithsen, pers. comm.). The density of *Polydora ligni*, a potential competitor, was extremely high in the 32× treatment during that period. *P. ligni* abundance was 5.7 times higher than *S. benedicti* in April ($\bar{x} = 56,246$ *vs.* $9,868/m^2$), 10.6 times higher in May ($\bar{x} = 114,860$ *vs.* $10,854/m^2$), 8.7

times higher in June ($\bar{x}$ = 186,698 *vs.* 21,512/m^2), and 4.2× higher in July ($\bar{x}$ = 262,482 *vs.* 62,167/m^2). *P. ligni* densities were consistently lower than *S. benedicti* in the 8× enrichment tank during those months (J. P. Grassle, pers. comm.; J. Frithsen, pers. comm.). The 32× enrichment tank experienced anoxic conditions between 29 July and 3 August 1983 (Frithsen *et al.*, 1985). *P. ligni* densities in this tank declined. Following this event, *S. benedicti* increased to 346,359 individuals/m^2 by September, the highest density observed for this species during the 3-year experiment. *Mulinia lateralis* was consistently abundant in this treatment throughout the summer and fall of 1983 (Frithsen, pers. comm.).

P. ligni and *S. benedicti* were among the species which demonstrated the greatest numerical response to nutrient enrichment in 1983 and sewage enrichment in 1984. Both species filter organic particles from the water column and feed on surface deposits. Frithsen and Doering (submitted) showed that these two species can increase net sedimentation when they reach high abundances. *P. ligni* is larger than *S. benedicti,* has longer palps (pers. obs.), is more aggressive (Levin, 1981; Whitlatch, pers. comm.), and could easily outcompete *S. benedicti* for food at the densities observed in the 32× enrichment tank. These interactions may have contributed to the small size and lower fecundity exhibited by *S. benedicti* in the 32× treatment relative to the 8× enrichment. In addition, oxygen stress might have produced decreases in reproductive output. A similar anoxic event occurred in mid September, 1984, when the 8× sludge treatment went anoxic and all benthos died. Just prior to the anoxia *S. benedicti* had been the numerical dominant. Unfortunately, no reproductive data were collected at that time.

Temperature may influence the magnitude of the reproductive response given by *S. benedicti* to organic enrichment. At low temperatures in May 1983 (12°C), increased food supply led to increases in size of reproducing females (Fig. 1) and to increased brood size independent of body size (Figs. 2a, b). In August, when temperatures had risen to 20°C, enrichment effects were reflected in level of reproductive activity. Few or no reproductive females were collected in control tanks. In the 8× and 32× enrichments, reproductive activity was high but body size and brood size were comparable to May control levels (Table II). Younger ages of females, due to recruitment during the summer, and response to increased metabolic demands at higher temperature or lower oxygen levels, may have been responsible. In addition, six- to seven-fold increases in *S. benedicti* density (Table II) may have intensified intraspecific competition and resulted in lower brood sizes than observed in May. In laboratory manipulations of temperature and food regimes, Levin and Creed (in press) found that body size and brood size of North Carolina *S. benedicti* with planktotrophic development increased in response to cooler temperatures. In that study food level had no effect on quantitative reproductive characters but lower food levels decreased the proportion of females which reproduced. The experiment did not examine competition for food.

S. benedicti appears to have the ability to translate enhanced food supplies directly into increased reproductive output. Utilization of heterosynthetic yolk sources during vitellogenesis, by sequestering materials from the circulatory system (Eckelbarger, 1980), may be one means by which *S. benedicti* can transfer food rapidly and directly into eggs and offspring. This capability may be essential to opportunists which depend on ephemeral resources and which, through utilization of these resources, may even contribute to their own demise. *S. benedicti* with planktotrophic development mature rapidly. They disperse their young during a 10–21 day larval planktonic phase (Levin, 1984b) and thus are not dependent on temporal persistence of a specific habitat.

Food quality, particularly nitrogen content, may regulate polychaete growth and reproduction (Tenore, 1977; Tenore and Chesney, 1985). Nutrient addition treatments in the eutrophication experiment and sewage treatments in the sludge experiment exhibited elevated chlorophyll levels, and enhanced phytoplankton concentrations (Nixon *et al.,* 1984; J. Maugham, pers. comm.). The combination of sewage sludge and a rich phytoplankton supply clearly represents a high quality food source for *S. benedicti.* The 2–5-fold increases in brood size exhibited in the 8× and 4× sludge tanks (relative to controls) far surpass the increases observed in the nutrient enrichment treatments the previous year. In studies of the benthic dynamics in the nutrient and sludge treatments during summer 1984, Maughan (in prep.) found that *S. benedicti* densities increased in the sludge treatments but not in the nutrient treatments. Phytoplankton production was also lower in the nutrient treatments during summer 1984 and Maughan (in prep.) suggests that filtering activities of the abundant amphipods, molluscs, and polychaetes in these tanks were responsible. By August 1984 *S. benedicti* had also attained very high densities in the 1× sewage treatment (J. Maugham, in prep.). Percent organic carbon in the top cm of sediment of the 1×, 4×, and 8× sludge treatments during August (3.58, 2.52, and 2.92, respectively) was higher than in control tanks (2.16–2.26) or the 1×, 4×, and 8× nutrient treatments (2.05, 2.19, and 2.58, respectively). These data suggest that at equivalent loadings, the sewage yielded more food for deposit feeders than the nutrient additions. Other constituents of the sewage sludge, which were not present in the nutrient addition tanks, may have stimulated recruitment, growth, or reproduction in *S. benedicti.*

Streblospio benedicti response to organic enrichment in the form of rapid, large increases in brood size is not surprising, and may demonstrate one reproductive tactic underlying opportunistic population dynamics. The calanoid copepods *Acartia tonsa* and *A. hudsonica* responded to nutrient enrichments with increases in daily egg production, maximum length and dry weight (Sullivan and Ritacco, 1985). However, these increases were often not reflected in zooplankton abundance while increased brood size in *S. benedicti* was accompanied by elevated benthic densities. The polychaete response to food may also explain why we often see strong seasonal cycles in *S. benedicti* populations which actively reproduce all year (*e.g.,* Levin, 1984a). Increases in brood size during periods of increased food availability or cooling temperatures (Levin and Creed, in press) may cause dramatic recruitment peaks. It is not known whether the increased brood size exhibited by the small individuals in the sewage sludge tanks were accompanied by a decrease in reproductive output later in life. Release of larvae earlier rather than later in life is certainly adaptive for an opportunistic species. More work is needed to determine exactly what triggers population explosions in opportunistic species and how these explosions are achieved. In the case of enrichment opportunists such as *S. benedicti,* organic particulates are rapidly converted into both somatic tissue and offspring and order of magnitude increases in larval production may result.

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LABORATORY EVIDENCE FOR A SIZE REFUGE IN COMPETITIVE INTERACTIONS BETWEEN THE HYDROIDS *HYDRACTINIA ECHINATA* (FLEMMING) AND *PODOCORYNE CARNEA* (SARS)

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ABSTRACT

Size refugia from competition, whereby one organism may grow too large to subsequently be overgrown by a superior spatial competitor, have rarely been documented in marine benthic communities. Size-symmetrical and size-asymmetrical competitive interactions were established between colonies of two hermit crab-associated hydroids, *Hydractinia echinata* and *Podocoryne carnea,* to assess the outcome of competition for space between the two species and its possible size-dependence. In size-symmetrical interspecific contests, *P. carnea* overgrew and killed *H. echinata* in 100% of 74 observed encounters. In size-asymmetrical contests in which *H. echinata* was always the larger colony, *P. carnea* was able to overgrow *H. echinata* in only 55% of 76 contests. *H. echinata* reaches a size refuge from overgrowth by *P. carnea,* but this "safe" size depends on the position occupied by a colony of *H. echinata* on a substrate with respect to physical or biological barriers to growth.

The outcome of intraspecific competition for space between *P. carnea* colonies depends on the relative growth rates of the competitors. In 23 intraspecific contests, the *P. carnea* colony with the highest rate of stolonal growth was always competitively dominant, and also overgrew *H. echinata* most rapidly in the size-symmetrical interspecific encounters. The ability of *P. carnea* to overgrow *H. echinata* in size-asymmetrical contests, however, did not depend on the growth rate of the *P. carnea* colony.

Data on the distribution and abundance of these two species suggest that *P. carnea* recruits to hermit crab shells at a low frequency and is thus a rare member of the hermit crab epifaunal community. The observed differences in interspecific competitive ability may reflect asymmetry in the frequencies with which these species encounter one another. The probability that a colony of *H. echinata* will encounter *P. carnea* is low, hence there will be little selection for interspecific competitive ability in *H. echinata*. The probability that a colony of *P. carnea* will encounter the common *H. echinata* is high; *P. carnea,* therefore, should maintain a mechanism for recognizing and overgrowing this important spatial competitor.

INTRODUCTION

The outcome of interactions between organisms does not remain constant throughout their lives. Encounters between competitors or between predator and prey may be sensitive to the relative sizes of the interacting individuals, and the effect one species has on another consequently may change throughout the ontogeny of those species (Buss, 1980; Werner and Gilliam, 1984). The dependence of many predator-prey interactions on prey size has been well documented in marine benthic communities: prey organisms which by chance escape predation when small may eventually

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grow too large to be consumed by a given predator (Ebling *et al.*, 1964; Paine, 1965, 1976; Dayton, 1971; Birkeland, 1974; Connell, 1975). The ability of some individuals in a population to reach a size refuge from predation may contribute significantly to the maintenance of community diversity and structure (Connell, 1975; Paine, 1976, 1977).

Few studies have examined how the outcome of competitive interactions between species may change as the relative sizes of the competing individuals vary. In several examples with colonial marine invertebrates, the direction of interspecific overgrowth has been shown to depend on the size (thickness) of the competing colonies: species A usually overgrows species B when A is larger, and *vice versa* (Day, 1977; Buss, 1980; Russ, 1982). Size refugia, where an individual of species B eventually reaches a size at which an individual of species A will be unable to overgrow it, may occur when the outcome of competition between two species is size-dependent (Buss, 1980). Size refugia from competition in benthic marine communities have been demonstrated by Buss (1980), in a system involving two bryozoans and a coralline alga in Panama, and by Sebens (1982), in a study of competition between the soft coral, *Alcyonium siderium,* and the compound tunicate, *Aplidium pallidum,* in the Gulf of Maine. In this study, I provide evidence for another example of an inferior spatial competitor, the athecate hydroid *Hydractinia echinata* (Flemming), reaching a size at which it can no longer be overgrown by an otherwise competitive dominant, the closely related hydroid, *Podocoryne carnea* (Sars).

In Long Island Sound and other Atlantic soft-bottom coastal areas, pagurid hermit crabs are extremely abundant, and the gastropod shells they occupy represent a source of hard substratum which supports a unique encrusting fauna (Karlson and Cariolou, 1982; Karlson and Shenk, 1983). *Hydractinia echinata* is one of the most common epifaunal species found on pagurid shells in Long Island Sound; *Podocoryne carnea* is present at much lower frequencies in this community. *H. echinata* and *P. carnea* display aggression towards one another, and in a previous examination of interspecific competitive ability *P. carnea* was shown to overgrow *H. echinata* consistently (Gallien and Govaere, 1974). However, all interactions examined were grossly size-asymmetrical, with a small explant of *H. echinata* placed in contact with a large *P. carnea* colony. This particular combination of colony sizes is only one of many conditions under which colonies may contact one another in natural encounters. The outcome of interspecific competition for space between *H. echinata* and *P. carnea* is examined further here, in both size-symmetrical and *Hydractinia*-biased size-asymmetrical interactions, to determine if *P. carnea* remains the superior spatial competitor across a range of size-specific encounters.

Natural history

Although the zooid morphology differs little between the genera (Goette, 1916; Mills, 1976), *P. carnea* and *H. echinata* display different patterns of basal tissue growth across a substratum. Growth of a colony of *H. echinata* is regulated by two interacting processes, elongation of stolons and expansion of ectodermal mat tissue (Fig. 1a). Stolons branch and anastomose to form intricate networks adherent to the substratum. Mat tissue, which consists of interconnecting gastrovascular canals and interstitial cells sandwiched between ectoderm, grows as a continuous sheet. As the mat tissue expands, it incorporates existing stolons into its structure. The interaction of the growth rates of mat tissue and stolons, combined with factors such as stolon branching frequency, give each colony of *H. echinata* a characteristic growth morphology during ontogeny (McFadden *et al.*, 1984). There is considerable genetic variation in stolon

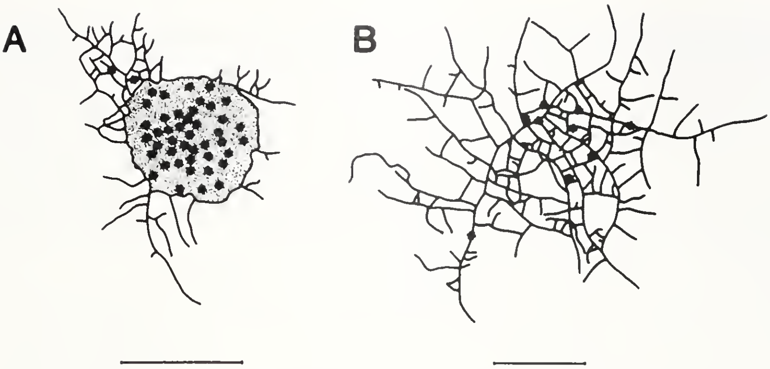

FIGURE 1. Camera lucida tracings of 17-day-old colonies, comparing the growth morphology of *H. echinata* (A) with that of *P. carnea* (B). *H. echinata* produces mat tissue (stippled area) and stolons during colony ontogeny, while *P. carnea* covers the substratum with a stolon network only. Asterisks indicate the positions of feeding polyps. Scale bars = 5 mm.

production: colony morphologies range from "stolonless" colonies which produce little or no stolon tissue as they grow, to "stoloniferous" colonies which form extensive stolon networks throughout colony ontogeny (Schijfsma, 1939; McFadden *et al.*, 1984).

Unlike *H. echinata, P. carnea* produces no mat tissue, but covers the substratum with an extensive stolon network (Fig. 1b). This network increases in density by continued proliferation of stolons; adjacent stolons eventually fuse laterally to form a basal crust analogous to the mat of *H. echinata* (Braverman, 1963, 1971, 1974; Braverman and Schrandt, 1966, 1969).

The hyperplastic growth reaction which occurs when genetically unrelated colonies of *H. echinata* contact one another is described in detail elsewhere (Hauenschild, 1954, 1956; Mueller, 1964; Ivker, 1972; Buss *et al.*, 1984). Briefly, nematocyst-bearing hyperplastic stolons arising from both colonies intertwine to form an extensive tangle in the area of contact (Buss *et al.*, 1984), and one colony will eventually overgrow and kill the other (Ivker, 1972). Competitive dominance is strictly transitive (Ivker, 1972), and highly correlated with growth morphology; in size-symmetrical encounters, colonies with high stolonal growth rates ("stoloniferous") predictably defeat colonies with slowly growing or no stolons ("stolonless") (Buss and Grosberg, in prep.).

The competitive overgrowth reaction of *P. carnea* is very similar to that of *H. echinata* (Tardent and Buhrer, 1982). Upon contact with a conspecific, stolons raise off the substratum and arch over the neighboring colony, producing a tangle of hyperplastic stolons in the area of contact. Scanning electron micrographs of *P. carnea* hyperplastic stolons show numerous discharged nematocyst threads, indicating that intraspecific overgrowth occurs by the same mechanism in the two genera. Transitivity and morphological correlates of intraspecific competitive ability between colonies of *P. carnea* will be examined briefly here, prior to discussion of interspecific competition.

MATERIALS AND METHODS

The colonies of *H. echinata* and *P. carnea* used in laboratory competition experiments were collected in August 1981 from the shallow subtidal (−3 m) gravel-mud bottom adjacent to No Man's Island, Old Quarry Harbor, Guilford, Connecticut. Individuals of *Pagurus longicarpus* with hydroid-encrusted shells were collected as encountered using SCUBA and were transported to the laboratory, where small pieces of ectodermal tissue containing 1–3 feeding polyps were excised from each shell. These tissue explants were placed on Plexiglas culture slides and held down by a loop of thread tied around the slide (Ivker, 1972). After 2–3 days, explants attached to the Plexiglas, and the thread was removed. This technique was used for all clonal propagations referred to in this paper. Colonies were maintained at approximately 20°C in recirculating natural seawater, and were fed for 2 h daily with day-old *Artemia* nauplii. Colonies were returned to clean seawater immediately after each feeding. Each slide was brushed weekly with a fine camel's-hair paintbrush to prevent the accumulation of growth-inhibiting detritus.

The use of clonal organisms facilitates an examination of size-specific competitive interactions, because encounters between individuals of the same genotype can easily be replicated over a wide range of size relationships. The competitive ability of an individual which is killed by a competitor when small can nonetheless be examined when the same individual is large by using a clonal replicate, whereas the competitive ability of a non-clonal organism which is killed when small can never be assessed at a larger size. In addition, the degree to which the outcome of a competitive interaction is due to genotypic variation in the competitive ability of the individuals involved can be separated from strictly size-dependent effects by pairing any one individual (genotype) with numerous competitor genotypes.

Intraspecific competition between colonies of P. carnea

Size-symmetrical competitive interactions were initiated between all possible pairwise combinations of four genotypes of *P. carnea* (labeled P1, P2, P3, P4). [The assumption has been made that each field-collected colony represents a unique genotype; potential difficulties with this assumption are discussed in detail in McFadden *et al.* (1984).] Single polyp explants of each of two colonies were established approximately 2 cm apart on Plexiglas slides and allowed to grow into contact with one another. The number of replicates of each pairwise combination varied from 2 to 6, due to difficulties experienced getting explants of some genotypes to attach successfully to slides. The interactions were observed at approximately weekly intervals until tissue of one of the two colonies could no longer be discerned on the slide. In addition, three replicate clones of each of the four genotypes were established as controls to determine colony morphology and growth rate in the absence of competitive interactions. Each control colony was traced at 3-day intervals over a period of 17 days, using a camera lucida attachment to a Wild dissecting microscope at 7.5×. Drawings were digitized using an image analysis system [Measuronics Corp., Linear Measuring Set (LMS)] to determine total length of stolons present at each date. Cumulative growth curves were plotted for each colony, and the slope of the linear regression of the log-transformed curve [log (mm stolon) = m log (time)] was used as an index of stolon growth rate. For further discussion of this method for fitting growth curves, see McFadden *et al.* (1984).

Interspecific competition: size-symmetrical contests

Size-symmetrical contests were initiated between 20 genotypes of *H. echinata* (labeled H1-H20) and 4 genotypes of *P. carnea* (P1–P4) in all 80 possible pairwise combinations. Single polyp explants of each species were established 1 cm apart on Plexiglas slides and the interaction of the two colonies was observed at approximately weekly intervals until one colony had completely overgrown the other. Overgrowth was considered complete when no polyps remained in one of the two colonies. Several colonies died before contact had occurred, including all four replicates of H14; these pairs have been eliminated from the results.

Size-asymmetrical contests

Size-asymmetrical contests were established between clones of the same 20 genotypes of *H. echinata* and 4 genotypes of *P. carnea.* Single polyp explants of *H. echinata* were established on Plexiglas slides and allowed to grow undisturbed for a period of six weeks. At this time they were photographed to record size and general growth morphology, and a single polyp explant of *P. carnea* was then established approximately 1 cm from the periphery (mat edge or outermost stolons) of the *H. echinata* colony. All four clones of genotype H10 appeared unhealthy at the time *P. carnea* was introduced onto the slides and they were therefore eliminated from the experiment.

The interspecific interactions were observed over a period of seven months, at which time the experiment was terminated. Photographs taken of the *H. echinata* colonies at the time of attachment of the *P. carnea* explants were converted to line drawings using a camera lucida on a Wild dissecting microscope at 7.5× (McFadden *et al.,* 1984). The area of mat tissue and area covered by stolon network (the polygon determined by connecting the free tips of all stolons) were then digitized using an Apple II graphics tablet to yield estimates of the size and morphology of each *H. echinata* colony just prior to contact with *P. carnea.*

RESULTS

Intraspecific competition between P. carnea *colonies*

The outcome of intraspecific competition between colonies of *P. carnea* is highly transitive, and can be predicted by stolon growth rate (Table I). P3, the colony with the fastest stolon growth rate, is the competitive dominant; P1, the colony with the slowest growth rate, is consistently overgrown by all other genotypes. Out of 23 contests, the only outcome which deviates from this dominance hierarchy is one of the 3 contests between genotypes P3 and P4. It is possible that the identities of the two colonies on

TABLE I

Mean duration (in days) of intraspecific contests between four genotypes of P. carnea

			Winning genotype			
		Growth rate*	P1	P4	P2	P3
Losing genotype	P1	(1.007)	—	86 (2) S.D. = 42	67 (4) S.D. = 17	70 (6) S.D. = 24
	P4	(1.062)	—	—	122 (2) S.D. = 6	131 (2) S.D. = 19
	P2	(1.319)	—	—	—	179 (6) S.D. = 30
	P3	(1.622)	—	118 (1)	—	—

* Slope of linear regression fit to the log-transformed cumulative growth curve [log(mm)/log(day)]. Numbers in parentheses indicate number of replicates.

this slide were reversed during the experiment; all other contests involving either P3 or P4 yielded results consistent with predictions based on a transitive hierarchy.

The amount of time required for one *P. carnea* colony to overgrow another depends only on the identity of the losing colony in the contest (Table I; Kruskal-Wallis, within rows, $P < .01$), and not on the identity of the winner (Table I; Kruskal-Wallis, within columns, $P = .42$). For instance, there was no difference in the number of days required for genotypes P2, P3, and P4 to overgrow inferior competitor P1, while the amount of time P3, the competitive dominant, took to overgrow P1, P2, and P4 was highly variable (Table I).

Interspecific competition

H. echinata exhibits little or no hyperplastic growth upon contact with *P. carnea.* Occasionally the growing tips of *H. echinata* stolons swell and rise off the substratum slightly in response to contact with *P. carnea,* but further hyperplastic development rarely occurs. Stolons of *P. carnea* rapidly overgrow *H. echinata* stolons without becoming hyperplastic, but begin hyperplastic growth immediately upon contact with mat tissue. *P. carnea* stolons are unable to grow across mat tissue, and consequently, mounds of hyperplastic stolon up to 5 mm in height accumulate at the periphery of the mat area at every point of contact between *P. carnea* stolons and *H. echinata* mat tissue (Fig. 2). These mounds may extend out over the surrounded mat tissue for as much as 2 cm, but they are not anchored to the underlying mat tissue and are easily broken off. The underlying *H. echinata* polyps are resorbed subsequent to overgrowth

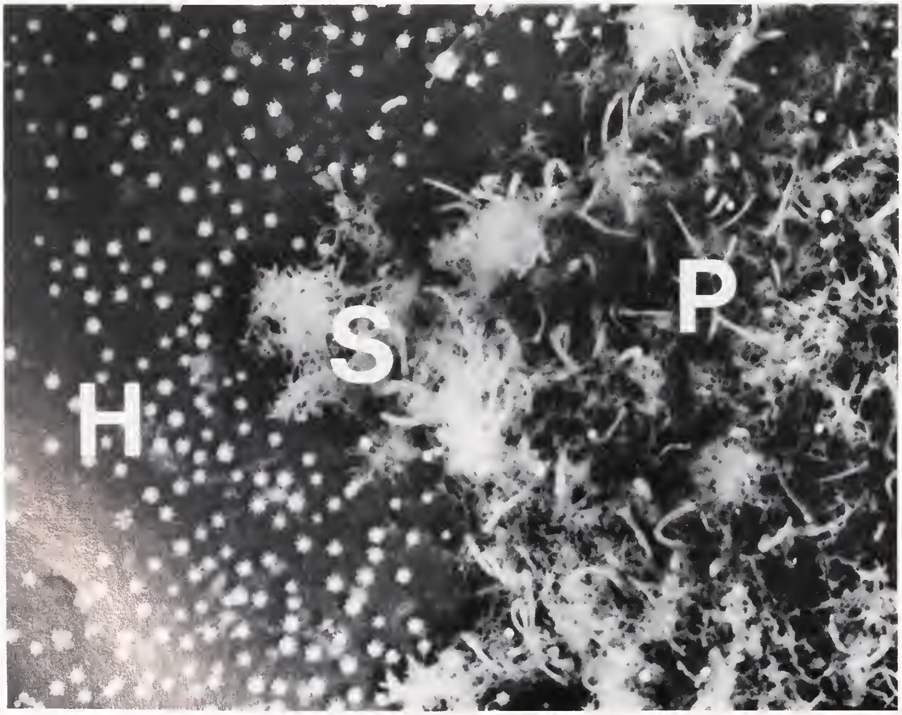

FIGURE 2. A contest between *P. carnea* (P) and *H. echinata* (H). *P. carnea* has produced hyperplastic stolons (S) where it is in contact with *H. echinata* mat tissue.

by *P. carnea* hyperplastic stolons, but if polyps remain alive elsewhere in the colony, the overgrown mat tissue can remain alive. If hyperplastic tissue is removed from the overgrown areas of mat, new polyps may be regenerated on the exposed mat tissue, as is also possible in cases of intraspecific overgrowth in *H. echinata* (Ivker, 1972). Death of the overgrown *H. echinata* colony occurs only when *P. carnea* hyperplastic stolons cover the entire surface of the colony and all the polyps have been resorbed, presumably curtailing nutrient intake and leading to starvation.

Size-symmetrical interspecific contests

In every one of the 74 size-symmetrical contests, *P. carnea* overgrew and killed *H. echinata* (Table II). The mean time for complete overgrowth was 37 days, although several *H. echinata* colonies survived for considerably longer. Genotype H12 withstood overgrowth by P4 for 161 days, eventually with just a single polyp protruding through *P. carnea* hyperplastic stolons. There is a significant association between the duration of a contest and the genotype of the *P. carnea* colony (Kruskal-Wallis, $P < .02$). P3, the fastest growing *P. carnea* genotype, overgrew *H. echinata* in the least time ($\bar{X} = 26$ days), whereas P4, one of the two *P. carnea* genotypes with the slowest growth rates, took the longest time to overgrow *H. echinata* ($\bar{X} = 46$ days) (Table II).

The growth morphology of the *H. echinata* colony does not affect the rate at which it is overgrown by *P. carnea*. Genotypes of *H. echinata* were categorized into three groups based on growth morphology: stolonless, stoloniferous, or intermediate. Morphological category was determined by previous quantitative measures of growth of these genotypes (McFadden *et al.*, 1984), as well as evaluation of the growth of each clone during the experiment. The duration of a contest between *P. carnea* and *H. echinata* did not differ significantly between these three morphological groups (Kruskal-Wallis, $P > .21$).

Size-asymmetrical interspecific contests

Small *P. carnea* successfully overgrew and killed large *H. echinata* in 42 of the 76 size-asymmetrical contests (Table III). In no cases did *H. echinata* overgrow *P. carnea*. The other 34 contests were terminated after approximately 235 days, at which time both species still occupied space on each slide, but the boundary between colonies, delineated by *P. carnea* hyperplastic stolons, had remained static for 2–3 months in all cases. All of these "standoffs" reflected identical situations: *P. carnea* had covered all areas of the slide which were initially vacant or occupied by *H. echinata* stolons, but was unable to overgrow living *H. echinata* mat tissue. Both colonies were substrate-limited by the presence of the other, and further growth could take place only if one colony died or resorbed tissue in the zone of contact.

Unlike the symmetrical contests, there was no significant relationship between the genotype of the *P. carnea* colony and the length of time it took to overgrow *H. echinata* in those asymmetrical contests which ended in the death of the *H. echinata* colony (Kruskal-Wallis, $P > .47$). There was also no significant difference between *H. echinata* morphological types when genotypes were pooled into the categories stolonless, stoloniferous, and intermediate (Kruskal-Wallis, $P > .75$). However, if absolute size of the *H. echinata* colony is examined instead of this qualitative measure of morphology, a significant trend is apparent. Colony size was broken down into two separate measurements, total area covered by mat tissue and total area covered by stolon networks; colony thickness does not change as colony area increases. It has been shown that the growth rates of mat tissue and stolons are largely independent of one another (Mc-

TABLE II

Number of days H. echinata *colonies survived in size-symmetrical contests*

	H. echinata colony																					
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	$\bar{X}$	S.D.
Morphology:	L	I	S	S	S	I	S	I	L	I	I	L	L	L	L	S	I	S	I	S		
P. carnea colony																						
P1	43	32	26	32	27	32	32	32	39	62	87	32	16	—	74	32	32	35	74	29	40	19
P2	13	32	35	29	32	35	62	37	27	25	32	32	32	—	62	20	—	32	70	32	36	14
P3	21	23	35	20	29	16	16	20	32	32	16	35	22	—	28	—	32	32	35	24	26	7
P4	32	32	20	35	32	35	37	27	23	58	35	161	32	—	79	16	51	27	95	51	46	34

L = stolonless, I = intermediate, S = stoloniferous.

TABLE III

Number of days H. echinata *colonies survived in size-asymmetrical contests*

	H. echinata colony																			
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20
Morphology:	L	I	S	S	S	I	S	I	L	I	I	L	L	L	L	S	I	S	I	S
P. carnea colony																				
P1	182	120	122	A	A	59	A	66	94	—	A	67	67	107	79	A	192	158	A	A
P2	182	117	122	213	A	25	A	48	55	—	158	51	67	67	A	A	A	A	A	A
P3	159	59	A	A	A	25	A	55	55	—	A	56	51	90	107	A	192	A	137	A
P4	187	A	A	187	A	59	A	A	109	—	A	112	51	154	112	A	A	A	A	A

'A' indicates the *H. echinata* colony was alive at the end of the experiment. L = stolonless, I = intermediate, S = stoloniferous.

Fadden *et al.*, 1984), and consequently, *H. echinata* survival time was tested with respect to each measure separately. There is no significant correlation between the area covered by *H. echinata* stolons at the onset of interspecific contact and the length of time the colony withstood overgrowth by *P. carnea* (Kendall tau, r = .12, $P > .15$, n = 42), although survival time appears to be generally longer for colonies with large stolon networks (Fig. 3).

There is, however, a significant correlation between the length of time a genotype of *H. echinata* withstood overgrowth and the mat area of the colony at the onset of the interaction (Kendall tau, r = .74, $P < .001$, n = 42) (Fig. 4). There appears to be a survival threshold at a mat area of approximately 100 mm^2. The frequency with which *H. echinata* colonies were overgrown by *P. carnea* is significantly higher among colonies with an initial mat area less than 100 mm^2 than among those in two larger size classes, 100–200 mm^2 and >200 mm^2 ($\chi^2 = 17.8$, df = 2, $P < .005$) (Table IV). The survival rate of colonies does not differ between the two upper size classes ($\chi^2 = 0.03$, df = 1, $P > .80$). *H. echinata* colonies with small initial mat areas were usually killed after *P. carnea* had completely surrounded them and built up enough hyperplastic stolons around their periphery to extend completely across the mat. *H. echinata* colonies with large initial mat areas could not be surrounded by *P. carnea* due to their size and to the confines of the culture slides, and hence could not be overgrown.

DISCUSSION

The position occupied by a *H. echinata* colony on a limited substratum may significantly affect the outcome of competition between a colony of this species and

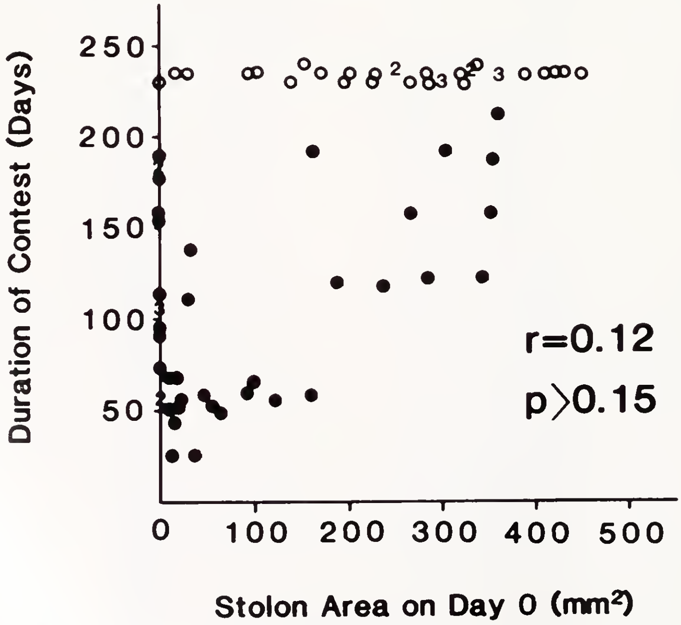

FIGURE 3. Correlation of the area covered by *H. echinata* stolons at the onset of an asymmetrical interspecific contest and the length of time the colony survived before being overgrown by *P. carnea*. Open circles represent colonies which remained alive at the end of the experiment. Numbers indicate multiple observations.

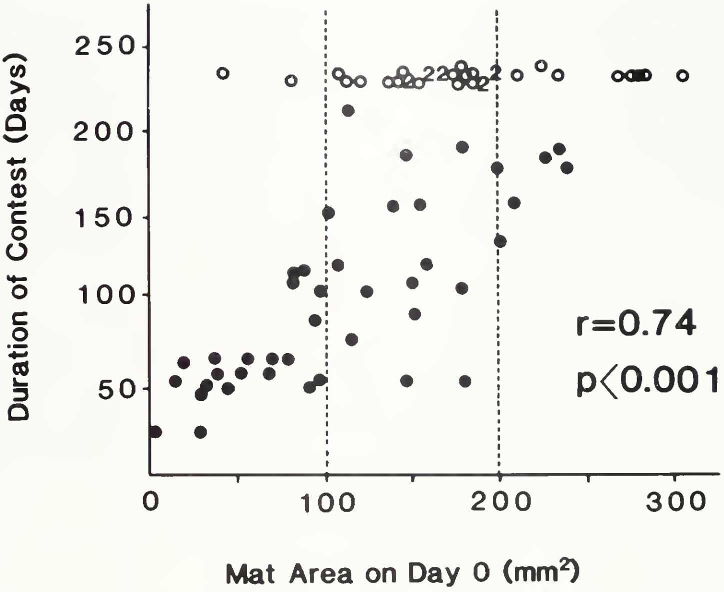

FIGURE 4. Correlation of the area of *H. echinata* mat tissue at the onset of an asymmetrical interspecific contest and the length of time the colony survived before being overgrown by *P. carnea*. Open circles represent colonies which were still alive at the end of the experiment. Numbers indicate multiple observations. Dashed lines demarcate the three size classes compared in Table IV.

one of *P. carnea*. The results presented above suggest that the ability of *P. carnea* to overgrow large *H. echinata* may depend on *P. carnea* first surrounding its competitor. Whether this condition can be met will be a function not only of the size of the *H. echinata* colony at the onset of the interaction, but also of the size of the free substratum, the position of the *H. echinata* colony relative to substrate boundaries, and the differential growth rates of the two species. Colonies with mat areas greater than 100 mm^2 at the time *P. carnea* was introduced reached the edges of the culture slides before *P. carnea* was able to surround them with its stolon network, and hence survived. Colonies initially smaller than 100 mm^2 were not growing rapidly enough to reach the limits of the slide before being surrounded by *P. carnea*.

The mat area threshold of 100 mm^2 above which *P. carnea* overgrows *H. echinata* is significant only in the context of the particular substrate employed in this study. In a substrate patch wider than 26 mm (the width of a Plexiglas slide), *P. carnea* should be able to surround and overgrow *H. echinata* colonies which are larger than 100 mm^2 at the onset of interspecific contact. A smaller substrate area, or the proximity of a

TABLE IV

Initial mat area (mm^2)	Number of *H. echinata* colonies overgrown by *P. carnea*	Number of *H. echinata* colonies surviving interaction
<100	21	2
100–200	16	23
>200	5	8

colony to a substrate boundary, will lower the size at which *H. echinata* colonies become safe from overgrowth. A colony which cannot be surrounded due to its proximity to a physical boundary will become safe from overgrowth at a size at which it could be overgrown were it instead surrounded by *P. carnea.* There will be a lower size limit to such a positional refuge: a very small *H. echinata* colony is likely to be overgrown by *P. carnea* regardless of its position on the substratum.

The interaction of colony size with position on the substratum may provide a refuge from competition on natural substrata. Many of the hermit crab shells on which *H. echinata* and *P. carnea* settle offer considerably less surface area than the slides used in this laboratory study. In addition, both the shell aperture and apex may operate as effective substrate barriers; a colony positioned along the aperture or around the apex of a shell will be difficult for another colony to surround. *H. echinata* planulae do recruit preferentially to points around the aperture of a shell (Teitelbaum, 1966). If new recruits of both species recruit simultaneously and in similar locations on the same shell it is likely that *P. carnea* will overgrow *H. echinata* and monopolize the substratum. If *P. carnea* recruits to a shell with an already established *H. echinata* colony, the result will be either overgrowth of the *H. echinata* colony and monopolization of the entire shell by *P. carnea,* or, alternatively, a "standoff" between the two species with maintenance of a static boundary between them (see Connell, 1976; Karlson, 1980).

The results of interspecific encounters in the laboratory suggest that, were all else equal, *P. carnea* should eventually competitively exclude *H. echinata* on hermit crab shells. However, data on the distribution and frequency of occurrence of these two species in Long Island Sound do not support this prediction. In all seasons, *H. echinata* is much more abundant than *P. carnea:* from June through October, 1982, only approximately 7% of all hydroid-encrusted shells collected were occupied by *P. carnea,* while *Hydractinia* colonies occupied the remaining 93% (Buss, Yund, and Harrison, in prep.). Competition, evidenced by hyperplastic stolons, occurs frequently between colonies of *H. echinata* which occupy the same shell. However, contact between *H. echinata* and *P. carnea* was observed only once from over 1000 hydroid-occupied shells collected (Buss, Yund, and Harrison, in prep.). This low rate of encounter between *P. carnea* and *H. echinata* is a product of the low frequency of occurrence of *P. carnea* combined with the probability that individuals of both species will colonize the same shell. Early competitive exclusion of *H. echinata* by *P. carnea* when both recruit to the same shell may also contribute to the low observed encounter rate.

Models of a two-species community in which one species is the dominant competitor predict that a population of the inferior species can be maintained if there is a concomitant difference in the recruitment ability of the two species such that the inferior competitor is able to recruit to unoccupied substrata more quickly or more reliably than the dominant competitor (Armstrong, 1976). In a system in which a size refuge from competition is in operation, a difference in rate of recruitment, or in the seasonal timing of recruitment, may enable the inferior competitor to grow to a safe size before the dominant competitor can recruit onto the open substratum (Sebens, 1982). Higher rates of post-recruitment mortality of the superior competitor, or frequent interference with its overgrowth ability by events such as partial predation, are additional mechanisms which would allow an inferior competitor to reach a size refuge before being overgrown.

There is some evidence that the failure of *P. carnea* to displace *H. echinata* on hermit crab shells, despite its competitive superiority, may be due to a low rate of recruitment of this species to hermit crab-inhabited shells. In Long Island Sound, recruits of *P. carnea* (small colonies occupying less than 30% of the shell surface) were found on only 0.2% of all shells collected from June to October, 1982, (n = 1663), whereas *H. echinata* recruits were present on 22% of these shells (Buss, Yund, and Harrison, in prep.). Because recruitment of *P. carnea* is apparently a rare event in this community, most *H. echinata* colonies will reach a size at which they are safe from overgrowth without ever encountering *P. carnea.* Yet the competitive superiority of *P. carnea* in interspecific contests ensures that an individual which does successfully recruit to a hermit crab shell can acquire and maintain space on that shell even if it is already occupied, or subsequently invaded, by *H. echinata.*

The asymmetry in the degree of interspecific recognition displayed by these two species can be interpreted in light of the rate at which they are likely to encounter one another in the field. *H. echinata* is one of the most abundant members of the hermit crab epifaunal community (Karlson and Shenk, 1983), and there is a high rate of encounter of this species with conspecifics and with other common epifaunal species, such as the bryozoan, *Alcyonidium polyoum* (Karlson and Shenk, 1983). However, the probability that an individual of *H. echinata* will encounter *P. carnea* during its lifetime is relatively low; consequently, there should be little selective pressure acting to maintain its ability to recognize this uncommon species as a potential competitor. As shown, *H. echinata* does not produce hyperplastic stolons in response to contact with *P. carnea. P. carnea,* on the other hand, has a high probability of encountering a *H. echinata* colony upon recruiting to a hermit crab shell, due to the high percentage of all shells which are occupied by this species. Selection should act on *P. carnea* to maintain a mechanism for recognition and subsequent deployment of competitive structures against this important interspecific competitor.

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FUNCTIONAL MORPHOLOGY AND SPECIES CHARACTERISTICS OF A LARGE, SOLITARY RADIOLARIAN *PHYSEMATIUM MUELLERI*

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ABSTRACT

Physematium spp. and related genera of radiolaria (*e.g., Thalassolampe* and *Actissa*) are characterized by a large, limpid spherical cell body varying in cytoplasmic compactness, but characteristically possessing numerous small (*ca.* 3 μm dia.) symbionts held in fine radiating axopodia surrounding the large central capsulum. An analysis of the cytoplasmic organization of *Physematium muelleri* suggests that this organism has adapted to a pelagic existence by increasing surface area to enhance prey capture while conserving biomass through the development of a large internally alveolate, spheroidal cell possessing fluid-filled spaces. The thin capsular wall is supported by a network of cytoplasmic strands emanating from the perinuclear region of the intracapsulum. The fine structural organization of the cytoplasm, composition and thickness of the central capsular wall, and the amount and kind of material deposited within the perinuclear envelope appear to be more significant taxonomic discriminating characteristics than the number or kind of siliceous spicules produced surrounding the central capsulum. The possible phylogenetic relationships among some genera related to *Physematium* and the functional morphology of the large, fluid-filled central capsule of close relatives are presented.

INTRODUCTION

Radiolaria are among the most abundant of biomineralizing Sarcodina occurring widely in the world's oceans. Their diverse and elegant siliceous skeletons have long attracted biological interest and have provided the main taxonomic characteristics used to classify radiolaria. In general, taxonomic criteria distinguishing species have included the overall shape of the skeleton, whether spherical, spiral, oval, or occurring as scattered spicules, etc. Further distinctions have been made on the number of concentric hollow spheres in the skeleton, or the geometry and arrangement of pores. Number and arrangement of surface spicules and spines also have been used widely in making taxonomic discriminations. The extensive use of skeletons as taxonomic indicators may be attributed to their elegant, geometric regularity and abundance in the sedimentary record (*e.g.,* Haeckel, 1887; Riedel, 1971). However, some species lack siliceous deposits or produce only few or scattered spicules in their peripheral cytoplasm making cytoplasmic morphology more significant in their taxonomy. Application of light and electron microscopic analyses of radiolarian cytoplasm has clearly contributed to finer taxonomic distinctions (*e.g.,* Hollande and Enjumet, 1953; Cachon and Cachon, 1977, 1985; Anderson, 1976, 1978a, b, 1983). The larger species producing little or no siliceous deposits have recently been investigated more extensively for their physiological and fine structural characteristics toward a more exact understanding

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of their functional morphology and phylogenetic affinities (*e.g.*, Cachon and Cachon, 1977; Anderson, 1978a, 1983; Swanberg and Harbison, 1980; Swanberg and Anderson, 1981; Anderson and Botfield, 1983). Some of these gelatinous, larger radiolaria reach diameters of several millimeters (Fig. 1), and are easily observed with the unaided eye and appear as opalescent, pale-green, or yellowish spheroidal bodies suspended in seawater.

Among these larger, gelatinous, solitary species of radiolaria, Haeckel (1887) described ten genera and forty-two species. The major diagnostic generic features he used were the presence or absence of alveoli within the large central capsule or in the peripheral envelope of cytoplasm known as the extracapsulum, and the occurrence and form of spicules in the extracapsulum. Of these species, *Physematium atlanticum* (Meyen, 1834) was one of the earliest of radiolaria identified. We have found this organism in great abundance near Barbados in December and January, and in the southern Sargasso Sea especially during the months of February and March, where it is often the commonest large solitary spumellarian in the surface waters. Unfortunately, the original description is rather ambiguous. Schneider's description (1858) of *P. muelleri* is unambiguous, however, and as it has priority over Haeckel's taxa, we have assigned our material to this species.

P. muelleri is characterized by a large, somewhat opalescent, spheroidal cell body (1–5 mm dia.) surrounded by a hyaline gelatinous layer making the total diameter of the organism 3–6 mm. Fine axopodia radiate peripherally through this gelatinous sheath. The nucleus was described by Haeckel as possessing a thick membrane surrounded by an alveolate intracapsulum with a thin capsular wall, though Cachon and Cachon (1977) have shown that the intracapsulum is not alveolate, based on fine structure evidence. The presence of C- or S-shaped, siliceous spicules in the extracapsulum distinguish *P. muelleri* from its close relative *Thalassolampe margarodes* based on the classical description. Brandt (1902) maintained that the presence of such isolated siliceous spicules were of limited systematic use at higher taxonomic categories, and then only when the soft body parts could be considered to be definitively different. He revised Haeckel's systematic scheme to place these genera together in the family Physematidae. He further observed that the cytoplasmic structure of *Physematium muelleri* and *Thalassolampe margarodes* agreed so closely as to make the latter genus superfluous; hence he recommended that the skeletonless genera be grouped with the older established genus *Physematium*. Modern researchers (Hollande and Enjumet, 1953; Cachon and Cachon, 1977; 1985) have accepted Brandt's familial designation but followed Haeckel's scheme in retaining the genus *Thalassolampe* for non-spiculate organisms.

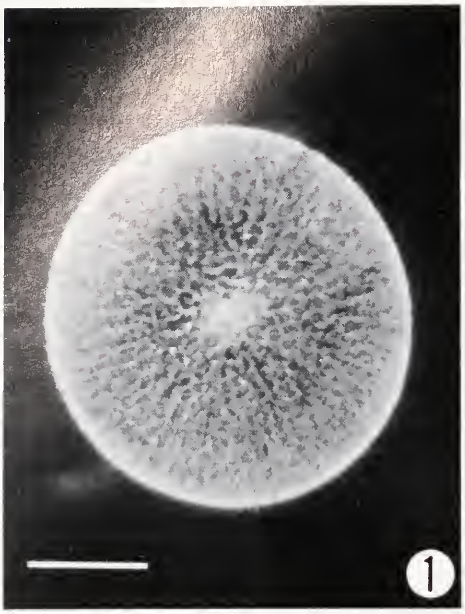
Although the spicules constitute a salient feature for systematic categorization, potentially, one of the most biologically significant characteristics of *Physematium* is the organization of the cytoplasm into a large gelatinous, spheroidal form with intracapsular lobes. These lobes vary in arrangement from a loosely packed anastomosing network with large peripheral vacuoles (as in *Physematium*) to more closely spaced lobes with numerous small peripheral vacuoles near the capsular wall (as in *Actissa*). The functional significance of this organization is examined in relation to host-algal symbiotic associations, prey apprehension, buoyancy functions, and possible phyletic relationships among related genera. To further elucidate the contribution of spicule number and morphology to the systematics of these large, gelatinous solitary radiolaria, we have examined the variation in spicule abundance and morphology in specimens collected by divers in open ocean locations near Barbados and in the Atlantic Ocean.

MATERIALS AND METHODS

Individual organisms were collected into hand-held glass jars by divers in the Caribbean Sea at a location approximately 1 mile from the west coast of Barbados, West Indies, during the month of January and at open ocean locations near Bermuda in the months of May and June, or during collection at sea in the North Atlantic Ocean (research cruises, OCEANUS 115 and 170, KNORR 53 and 94, ISELIN 83-1, and 83-11, CALANUS 85-5, and JOHNSON SEA-LINK). Specimens collected near Barbados were fixed for electron microscopy (Anderson, 1976), embedded in epoxy (LX 112), sectioned with a diamond knife, and ultrathin sections collected on uncoated copper grids. Sections were post-stained with Reynold's lead citrate and observed with a Philips EM 200 or EM 201 electron microscope operated at 60 kV. Other specimens were fixed immediately after capture using cacodylate-buffered 3% glutaraldehyde (pH = 7.8) and refrigerated for later examination by light microscopy and preparation for scanning electron microscopy. Specimens for scanning electron microscopic examination were rinsed in distilled water, immersed in 10% v/v ethanol to prevent large ice crystal formation during freezing, deposited on scanning microscope stubs, frozen in liquid nitrogen, and freeze-dried under vacuum. A Cambridge Stereoscan 250 Mk2 scanning electron microscope was used to examine the specimens.

RESULTS

Light-microscopic views of the whole organism (Fig. 1) exhibit the large centrally located nucleus, widely spaced lobes, and alveolate intracapsulum, enclosed by a thin capsular wall adorned with a thin layer of loose spicules (Fig. 2) of varying morphology but typically appearing as C- or S-shaped structures (*ca.* 100–150 μm in length). An overview of the organization of the central capsule is presented in the composite line drawing (Fig. 3) and low-magnification electron microscopic perspective (Fig. 4). The organization and position of the nucleus (N), the surrounding radially arranged lobes and their relationship to the thin peripheral capsular wall (CW) is illustrated in Figures 4 and 5. Numerous cytoplasmic strands (fusules) occur in the capsular wall and connect the intracapsular cytoplasm with the radiating rhizopodia penetrating the peripheral jelly layer. The intracapsular lobes are widely spaced and interconnected by thin strands of cytoplasm. Alveoli formed by large vacuoles surrounded by a thin layer of cytoplasm within the intracapsular lobes occur sporadically within the central capsular cytoplasm and more commonly at the perimeter near the capsular wall. Higher magnifications of the nucleus (Figs. 6, 7) exhibit the perinuclear perforated organic wall and extensions of the nucleus through the pores into the surrounding cytoplasmic lobes. The perinuclear organic wall exhibits a finely fibrous texture and is enclosed within a cisterna produced by cytoplasmic extensions of the intracapsular lobes (Arrow, Fig. 7). The nuclear membranous envelope is separate from the cisternal membrane enclosing the fibrous matter of the perinuclear wall and exhibits a structure typical for eukarayotic cells with transmembranous pores. The intracapsular lobes are richly supplied with mitochondria, Golgi bodies (Fig. 8), and electron-dense granules, frequently grouped with peroxisomes in clusters (Fig. 9). Groups of mitochondria are commonly observed encircling a cluster of electron-dense granules and smaller mitochondria (arrow, Fig. 4). Occasional peroxisomes are distributed at the periphery of the ring of mitochondria, and some of the peroxisomes encircle the mitochondria (Fig. 9) indicating a close structural and perhaps functional association. Large, less densely stained lipid droplets



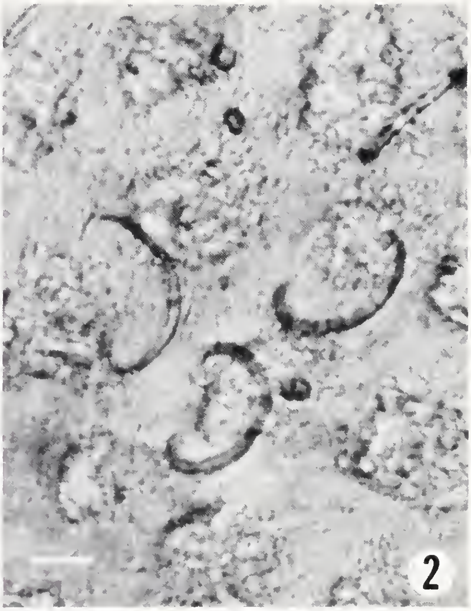


FIGURE 1. A living *Physematium muelleri* with prominent intracapsular lobes and a centrally located nucleus. Bar = 1 mm.

FIGURE 2. Siliceous spicules on the surface of the central capsular wall of *P. muelleri*. Bar = 20 μm.

are also observed in the regions of the cytoplasmic lobes where the ensembles of encircling mitochondria and dense granules are abundant.

The capsular wall possesses numerous fusules (strands of cytoplasm) directed outward, forming continuity between the intracapsular lobes and the extracapsular rhizopodial assembly (Fig. 5). The fusule structure resembles that in other large species of solitary radiolaria (*e.g.*, Anderson, 1976, 1983, p. 114), consisting of a thin strand of cytoplasm emerging from a tip of an intracapsular lobe, penetrating the capsular wall to which it is attached, and emerging on the extracapsular side as an electron-dense segment surrounded by a collar-like rim. The rim is perforated by micropores, giving a sieve-like quality to the wall of the rim. Distal portions of the extracapsulum exhibit rhizopodia and digestive vacuoles of varying diameter. Scanning electron microscopic views of the surface of the central capsule (Figs. 10, 11) show that the fusules occur in small clusters distributed over the surface of the central capsule. This is consistent with the transmission electron microscopic evidence showing long spaces of capsular membrane separating groups of fusules.

As a contribution to the comparative fine structure of *Physematium* and its relatives, we have examined the capsular organization of *Actissa* sp. collected at the same location near Barbados (Fig. 12). The peripheral capsular cytoplasm is more dense than that of *Physematium*, and possesses large peripheral vacuoles near the capsular wall as described earlier by light microscopic investigations (*e.g.*, Haeckel, 1887). These fine structural data confirm one of the distinguishing characteristics of the two species reported by Haeckel, *i.e.*, the presence of a thickened organic wall and large intracapsular spheroidal vacuoles in *Actissa*, and their absence in *Physematium*. Prominent outward directed collars (asterisk, Fig. 12) in the capsular wall of *Actissa* enclose the

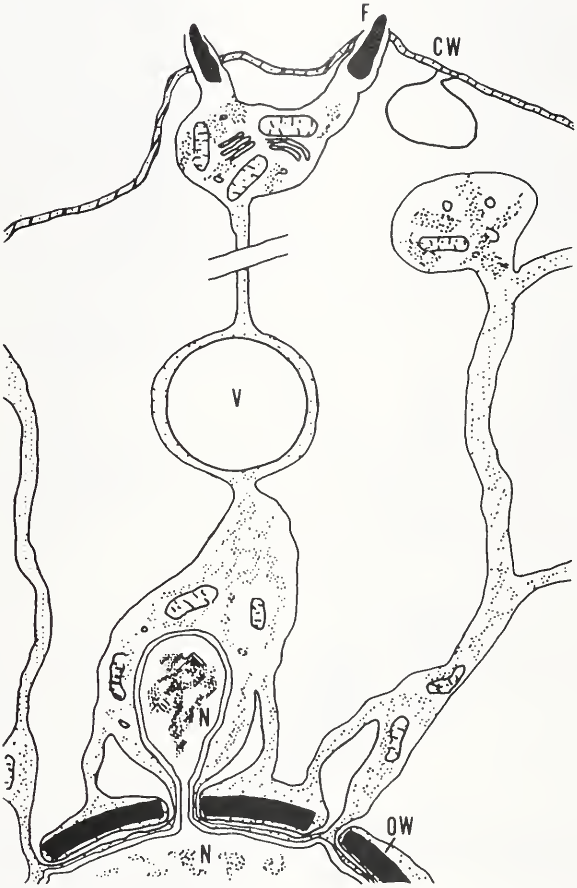
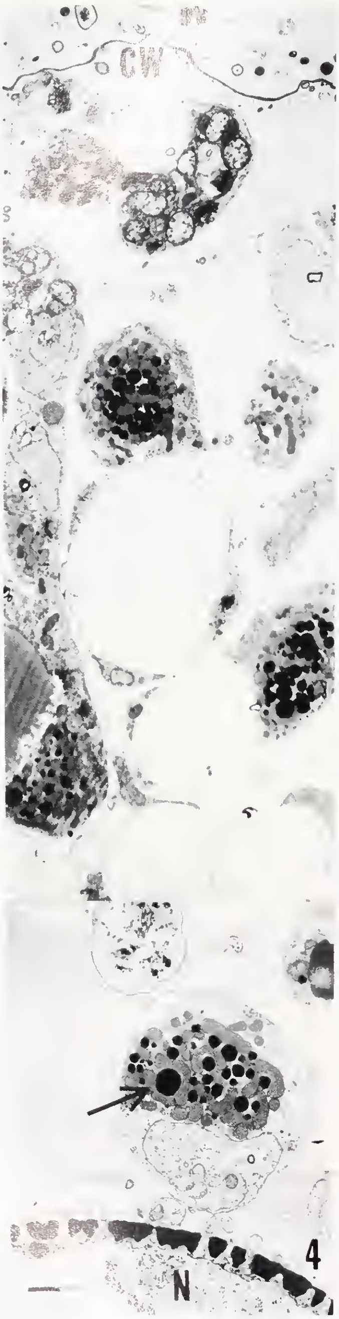


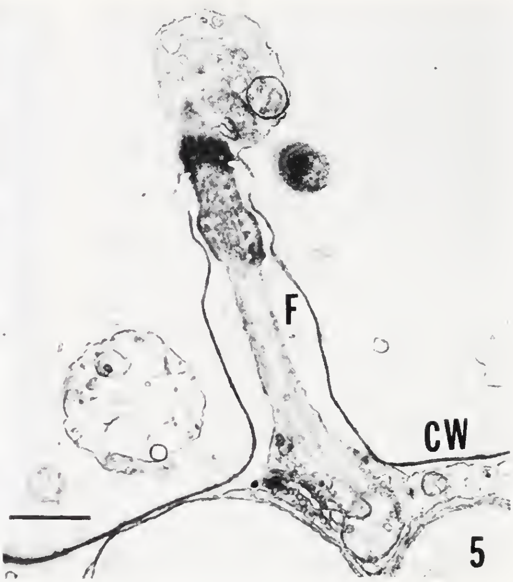
FIGURE 3. A line drawing illustrating a segment of the central capsular cytoplasm including the nucleus (N) with a thick organic wall (OW), radially arranged, widely spaced lobes with large vacuoles (V), and a thin peripheral capsular wall (CW) bearing the fusules (F) connecting the intracapsulum with extracapsulum.

fusule cytoplasmic strands. Numerous mitochondria and occasional segments of endoplasmic reticulum occur in the region proximal to the vacuolar layer.

Symbiont fine structure

We have observed very small yellow-green to yellow-brown symbionts (*ca.* 3–4 μm dia.), similar to those figured by Hollande and Enjumet (1953), in the extracapsular cytoplasm of both *Physematium* and *Actissa.* The fine structure of the symbionts (Fig. 13) indicates they are one of the Chrysophycophyta (eukaryotic yellow-green pigmented flagellates) with plastids composed of lamina with three thylakoids. The double membrane of the nuclear envelope encloses the plastids which are found occasionally in a parietal position within the cytoplasm. A granular pyrenoid within the plastid is penetrated by thylakoid membranes (Py, Fig. 13) and is prominently displayed in longitudinal sections. Mitochondrial lobes with tubular cristae and profiles of Golgi bodies





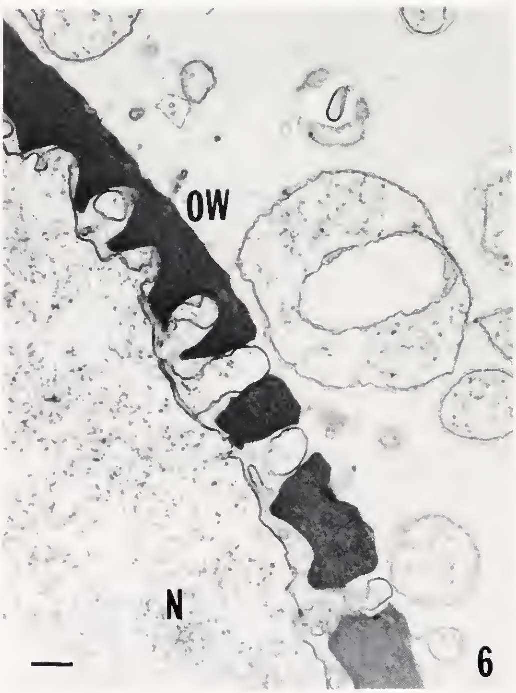
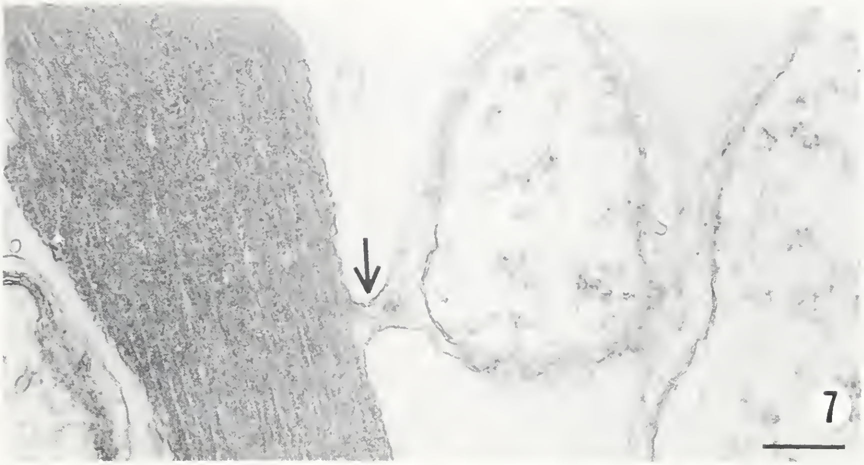
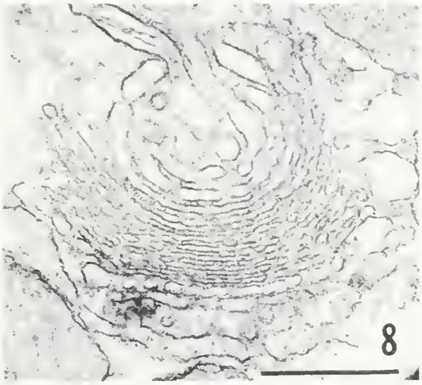


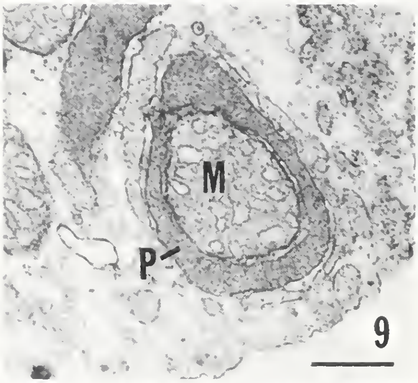
FIGURE 4. A montage of representative transmission electron microscopic sections along a radial dimension from the nucleus (N) to the capsular wall (CW) exhibits the thick, porous, nuclear wall, surrounding cytoplasmic lobes, and the peripheral thin capsular wall. Clusters of densely stained lipid deposits and mitochondria (arrow) occur abundantly in the intracapsular lobes. Bar = 5 μm.

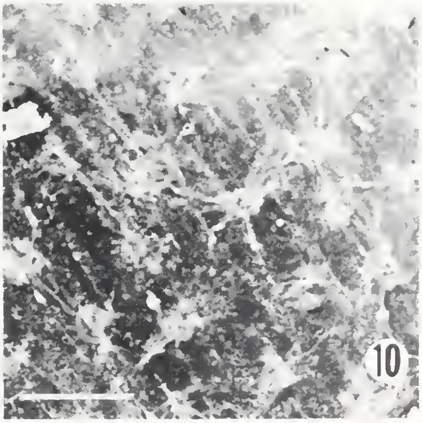
FIGURE 5. Fusule detail showing the protrusion of the capsular wall (CW) surrounding the cytoplasmic strand (F) projecting distally from the central capsule. Bar = 1 μm.

FIGURE 6. An enlarged view of the thickened wall (OW) surrounding the nucleus (N) with lobate projections of the nucleus in the pores and extending into the perinuclear space. Bar = 1 μm.









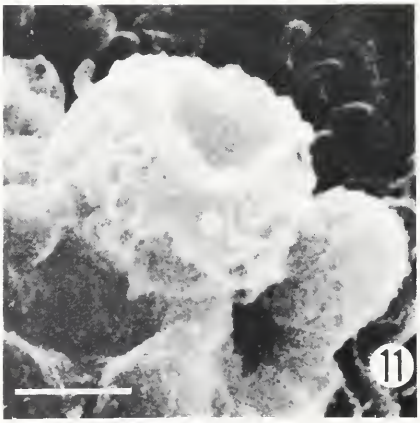


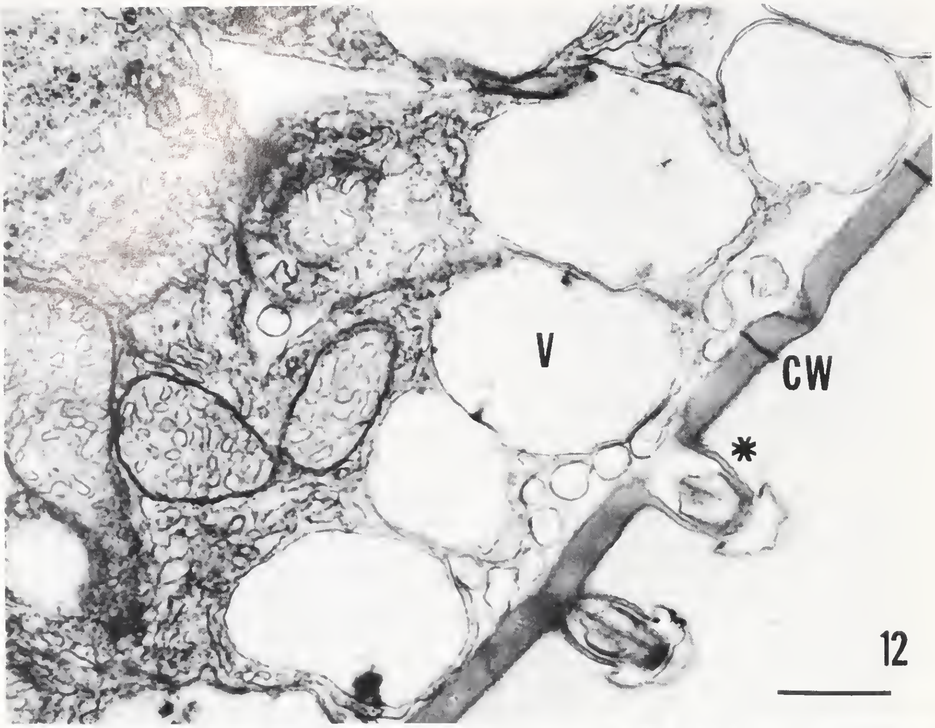
FIGURE 7. A detailed view of the organic wall surrounding the nucleus, showing the fine fibrillar quality of the organic substance in the wall and the enclosing living membrane (arrow) extending from a nearby cytoplasmic lobe. Bar = 0.5 μm.

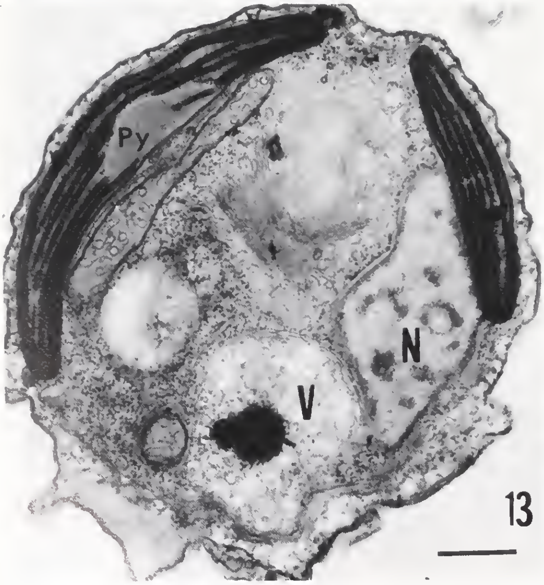
FIGURE 8. Golgi apparatus in a segment of an intracapsular lobe of *Physematium muelleri*. Bar = 0.5 μm.

FIGURE 9. A close spatial association occurs frequently between mitochondria (M) and peroxisomes (P) which sometimes encircle the mitochondria. Bar = 0.5 μm.

FIGURE 10. A scanning electron microscopic view of the surface of the central capsular wall showing the arrangement of fusules. Bar = 50 μm.

FIGURE 11. A higher magnification view of a fusule and surrounding rhizopodia on the central capsular wall. Bar = 1 μm.





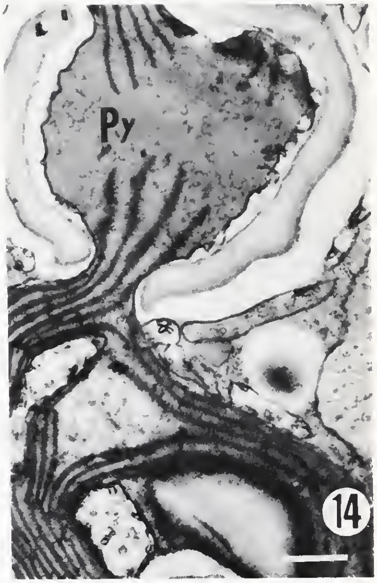


FIGURE 12. The peripheral intracapsular organization in *Actissa* sp. exhibits the large peripheral vacuoles (V) in the cytoplasm and thickened capsular wall (CW) with short fusules (asterisk) directed peripherally. Bar = 1 μm.

FIGURE 13. A section of a yellow-green pigmented algal symbiont (*ca.* 3 μm diameter) associated with *Physematium muelleri* and *Actissa* sp. The parietal plastids with internal pyrenoids (Py) are enclosed within the double membranes surrounding the nucleus (N). Storage vacuoles (V) are commonly observed in the symbionts. Bar = 0.5 μm.

FIGURE 14. The fine structure of the pyrenoid (Py) and its surrounding starch sheath within a dinoflagellate symbiont associated with *P. muelleri.* Bar = 0.5 μm.

are scattered throughout the central cytoplasm. Some symbionts possess a large excentrically located vacuole which is electron luscent or sometimes contains amorphous matter or densely staining granules (V, Fig. 13). We have not observed flagella, probably owing to the coccoid state of the algal cells as is typically observed in a symbiotic association with radiolaria (*e.g.*, Anderson, 1976, 1983; Anderson *et al.*, 1983). *Physematium* sp. also possess dinoflagellate symbionts (Fig. 14) resembling those previously observed in radiolaria (Anderson, 1976, 1983). In some cells, we have observed as many as three pyrenoids, while in previous observations of dinoflagellate symbionts (identified as *Amphidinium* sp.) there were either one or two pyrenoids.

Spicule abundance and morphological diversity

A sample of 60 specimens exhibiting a gross morphology of *Physematium* was examined to determine the abundance of spicules within the cytoplasm immediately surrounding the central capsular membrane. The abundance varied from no spicules to a few to hundreds and thousands per organism, suggesting an intergradation in spicule density among specimens. We suspect that spicule number may not be a good characteristic to distinguish species and therefore suggest that additional research is needed to evaluate Haeckel's assumption that spicule presence or absence is a species-specific trait. The absence of spicules may be due to a physiological state of the organism, rather than a genetic difference. A survey of 121 specimens of SCUBA-collected *Physematium muelleri* was made to determine the morphology of the spicules. The shape of the spicules was categorized as (1) straight needles, (2) C-shaped, (3) mixture of C-shaped and S-shaped, (4) mixture of C-shaped and straight, or (5) a mixture of shapes including all of the above and C-shaped forms with a small side branch. Fifty-four had simple straight spicules, 34 had C-shaped spicules, 5 had a mixture of C-shaped and S-shaped, 14 had a mixture of straight and C-shaped, and 14 had a mixture of heterogeneous shapes mentioned above. These data indicate that gradations in mixtures of spicule shape occur in specimens collected from the same locality, and that spicule type is probably not a good criterion for erecting separate species. The general intergradation of form of the spicules also exemplifies the remarkable heteromorphic variability in spicule composition of Radiolaria and raises the more general issue of the merit of using fine skeletal details in setting species boundaries.

Discussion

There is a component of arbitrariness inherent in all taxonomic criteria and the hierarchy of relative importance of various features is inescapably anthropocentric; this is particularly predominant in the systematics of radiolaria because of the salient aesthetic properties of their cytoplasmic and skeletal morphology. In recent publications, we have partially addressed the issue of taxonomic criteria and the appropriate kinds of attributes that may be most productive in developing a phylogenetically sound and heuristically valid taxonomic paradigm (Anderson, 1983, pp. 82–84; Swanberg and Anderson, 1985; Swanberg *et al.*, 1985, 1986). Our research on solitary and colonial radiolarian physiology (*e.g.*, Anderson, 1978b; Anderson and Botfield, 1983; Swanberg, 1983; Anderson *et al.*, 1985; Swanberg and Anderson, 1985) of *Spongodrymus* sp. and related spongiose skeletal solitary Spumellaria has given us an opportunity to examine in some detail a number of the larger, gelatinous Spumellaria including *Actissa, Physematium, Thalassolampe,* and *Thalassicolla.* A summary of our current understanding of the major morphological and fine structural features distinguishing these four genera is presented in Table I.

TABLE I

Major taxonomic characteristics of some larger gelatinous radiolarian genera

Taxonomic attribute	*Actissa*	*Physematium*[1]	*Thalassolampe*[1]	*Thalassicolla*
Cell dia. (mm)	0.2–1.5	1.0–12.0	2–15	1–6
Central capsule dia. (mm)	0.1–0.2	1.0–10.0	2–12	0.1–2.5
Cytoplasmic organization				
Nucleus	spherical, envelope thin, composed of double membrane	spherical, enclosed within thick organic wall within nuclear envelope	spherical, enclosed within thick organic wall within nuclear envelope	spherical, envelope thin membranous
Intracapsular lobes	closely grouped but with peripheral vacuoles near the capsular wall	forming a network of strands with fluid-filled, alveolate spaces	alveolate and widely spaced radial lobes	closely spaced radial lobes, dense cytoplasm
Capsular wall	thin organic wall within membranous envelope	thin, membranous, with organic deposit in cisterna	thin, membranous, with organic deposit in cisterna	thick organic wall in cisterna of capsular membrane
Extracpsular organization	thin, non-alveolate	thin, non-alveolate	thin, non-alveolate	robust, alveolate forming frothy cytoplasmic layer
Siliceous deposits	none	spicules few to many, of varied shape (S, or C-shaped)	none	none

[1] Note: See text, although these are separated in previous treatments, our data suggest these two genera may be merged.

Our evaluation of the fine structure of the cytoplasm of these organisms in relation to skeletal spicule variation has been informed by the observations of Hollande and Enjumet (1960, p. 66) decrying the poor systematic value of some skeletal variations, especially the significance of lattice versus spongiose skeletal morphology, and has led us to re-evaluate the importance of spicule abundance and morphology in erecting generic categories. This critical re-appraisal seems especially relevant to *P. muelleri* because of its unusual delicate spicules and the unique features of its large spheroidal central capsule including the very thin capsular membrane and the substantial perinuclear wall. We consider these features to be significant phyletically and taxonomically and representative of a functional morphology adapted to enhance buoyancy, algal symbiont associations, and possibly prey apprehension.

We present the first observation of a yellow-green pigmented chrysophyte-type alga in association with radiolaria. It is not immediately clear, however, why some individuals possess the yellow-green pigmented algal associates while others have dinoflagellate symbionts. Similar thin-walled cytoplasmic sheaths of host cytoplasm surround both kinds of algae, but we do not know if the physiology of the association, including kind and translocation rate of photosynthates from alga to host, is similar for the two types of algae.

The cytoplasmic organization of the larger gelatinous Spumellaria suggests a phylogenetic pattern of development progressing from an ancestral form resembling *Physematium* with a thick perinuclear wall and delicate vacuolated capsular cytoplasm toward *Actissa* with a thickened capsular wall, rather closely packed cytoplasmic lobes bearing numerous peripheral alveolate vacuoles, but still lacking extracapsular alveoli. At a more advanced stage, an organization more characteristic of modern *Thalassicolla* sp. may have emerged, with densely packed intracapsular lobes of cytoplasm, a thickened porous capsular wall, and a massive array of extracapsular alveoli.

Our fine structural analyses of *P. muelleri* show that the delicate intracapsular cytoplasm supporting the thin central capsular membrane provides a large increase in cell volume with moderate cytoplasmic elaboration. This delicate construction, while increasing surface area and conserving cytoplasmic mass, also leaves the nucleus relatively unprotected. The thickened perinuclear wall may provide protection for the nucleus suspended within the delicate web of anastomosing intracapsular lobes. The adaptive value of the radially arranged lobes with large intra-lobular spaces is not obvious. Observations of living specimens floating in the open ocean and in laboratory culture indicate that, like many gelatinous Spumellaria, these organisms are neutrally buoyant. This buoyancy may be attained by secretion of low density fluids within the free space among the lobes. The presence of a fluid within the central capsule has been confirmed by piercing the organisms in laboratory culture. In most cases, the pierced organisms exude the fluid, but do not burst. The capsular membrane eventually heals and the large inflated form is re-established.

The functional morphological significance of these features appears to be profound. An hypothetical spherical organism relying on density-dependent predation for food is under selective pressure to increase its ratio of surface area to volume with minimum expenditure of energy and maximum utilization of cytoplasmic mass (Anderson, 1985, Swanberg *et al.*, 1985). One way to accomplish this is to protrude thin lobes of cytoplasm either supported on delicate, elongate skeletal elements if present (Anderson, 1983, pp. 178–180, 1985; Swanberg *et al.*, 1985) or attached to a thin lamina such as the delicate capsular membrane to increase the associative strength while simultaneously keeping the amount of supporting surface cytoplasm at a minimum. Few skeletonless organisms appear to have employed this option as observed in *P. muelleri*. A large surface area for improved symbiont holding capacity and greater probability of prey apprehension, is produced while the large fluid-filled spaces between the delicate cytoplasmic lobes provide volume at low metabolic expense. Although the capsule contour remains spherical, the total cytoplasmic surface area is large relative to the smaller, metabolically active volume. The radiating delicate axopodia surrounding the globose central capsule are efficiently disposed to provide a large prey apprehending area.

Haeckel considered *Actissa* to be the most "primitive" of the radiolaria. Our observations suggest that in its ultrastructure *Physematium muelleri* is actually closer to a simple spherical cell and more primitive in its organization of the capsular cytoplasm than many Spumellaria. The adaptation to increased surface area at low metabolic cost is a major strategy for a number of groups of gelatinous metazoan predators such as Coelenterata and Ctenophora in the open ocean. If, indeed, *Physematium* is a primitive form of radiolarian, the evolution of a light-weight, large-surface-area morphology may have been an early adaptation that preceded massive skeletal deposition as a means of supporting and enhancing large cytoplasmic surfaces in these symbiont-bearing, opportunistic planktonic predators.

ACKNOWLEDGMENTS

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NEPHRIDIA IN THE LARVAE OF HEMICHORDATES AND ECHINODERMS

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ABSTRACT

The pore canal-hydropore complex in the larvae of echinoderms and hemichordates has long been recognized as an important character establishing a close phylogenetic relationship between the two phyla. An experimental and ultrastructural analysis of this complex in a tornaria and a bipinnaria larva indicates that it is a functional nephridium. The ciliated pore canal drives a constant, unidirectional efflux of coelomic fluid out of the hydropore. Two percent and 14% of the body volume are cleared per hour at the hydropore by a tornaria of *Schizocardium brasiliense* and a bipinnaria of *Asterias forbesi*, respectively. Fluid recovery by the coelom is from the blastocoel, the presumptive blood vascular space, across basal lamina and podocytes lining the coelomic cavity suggesting that the discharged fluid is formed by ciliary-driven ultrafiltration. Although invertebrate deuterostomes are believed to lack discrete excretory organs, an analysis of the metamorphosis of the larval nephridia suggests that adult echinoderms and hemichordates possess functional metanephridial systems.

INTRODUCTION

A signal achievement of classical morphology was the recognition of a close relationship between hemichordates and echinoderms based on structural similarities of their larvae (Gemmill, 1914; van der Horst, 1939). The most striking of these similarities is the pore canal-hydropore complex. It consists of a duct leading from a coelomic cavity to an external pore situated asymmetrically to the left of the dorsal midline as described in larvae of Enteropneusta and all five extant classes of echinoderms (Hyman, 1955, 1959). Yet despite the careful attention paid to this organ by morphologists of the nineteenth and early twentieth centuries, only one, T. H. Morgan, commented on its function. He speculated that "The whole structure is suggestive of an excretory arrangement [in the tornaria] . . ." (Morgan, 1894).

Invertebrate deuterostomes are generally believed to lack discrete nephridial organs and nowhere is the belief more frequently advanced than in the Echinodermata (Binyon, 1966; Barnes, 1980). We have been studying the generality of a model that predicts the occurrence of functional metanephridia in organisms where there is the potential for ultrafiltration of vascular fluid into a coelomic cavity, *viz.*, in coelomates with a blood vascular system (Ruppert and Smith, 1985, material in prep.). Our attention was drawn to larval echinoderms and hemichordates because of reports of a contractile vesicle beating rhythmically adjacent to the coelom and pore canal of definitive larvae in the Ophiuroidea (Gemmill, 1918), Echinoidea (Bury, 1896), Asteroidea (Gemmill, 1914), and Enteropneusta (Morgan, 1894). This organization suggested that blastocoelic fluid could be pressure filtered across the wall of the coelom and pore canal, modified by the lining cells, and released at the hydropore.

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Objectives of this investigation were to determine the pattern of fluid flow in the pore canal in the larvae of the asteroid, *Asterias forbesi*, and the enteropneust, *Schizocardium brasiliense*, and to identify potential sites of ultrafiltration using transmission electron microscopy (TEM). Because it soon became apparent that the larval complexes functioned as nephridia, another objective was to re-examine the adult derivatives of the larval structures to determine if adult enteropneusts and echinoderms might express functional nephridia.

MATERIAL AND METHODS

Collections of adult animals were made in March 1985 from a rock jetty at Murrell's Inlet, South Carolina (*Asterias*), and from a mudflat at North Inlet near Georgetown, South Carolina (*Schizocardium*) (Fox and Ruppert, 1985). Tornaria larvae of *Schizocardium* and bipinnaria larvae of *Asterias* were reared from eggs spawned in the laboratory. Cultures were maintained under constant agitation at 16°C for approximately 6 months with thrice weekly changes of natural seawater (33‰) and the addition of a few squirts from a Pasteur pipet of *Dunaliella salina* and *Isochrysis galbana*, as food.

Flow direction and flow rates in the ciliated pore canal were determined by microperfusion of the adjoining coelom with 0.45 μm latex microspheres (Polysciences), stabilized with BSA (Sigma), and suspended in seawater. These were introduced through the hydropore without damage to the larva. Particle movements were observed directly under a compound microscope (Zeiss Photomicroscope I) and recorded using cinemicrography. Cinemicrographic sequences were obtained on 16 mm film (Kodak Plus-X, Double-X, and Ektachrome 7240 color positive film) at 16 fps. Analyses of particle movement were made from prints of selected sequences and with the aid of a motion analyzer (Athena model NT-O).

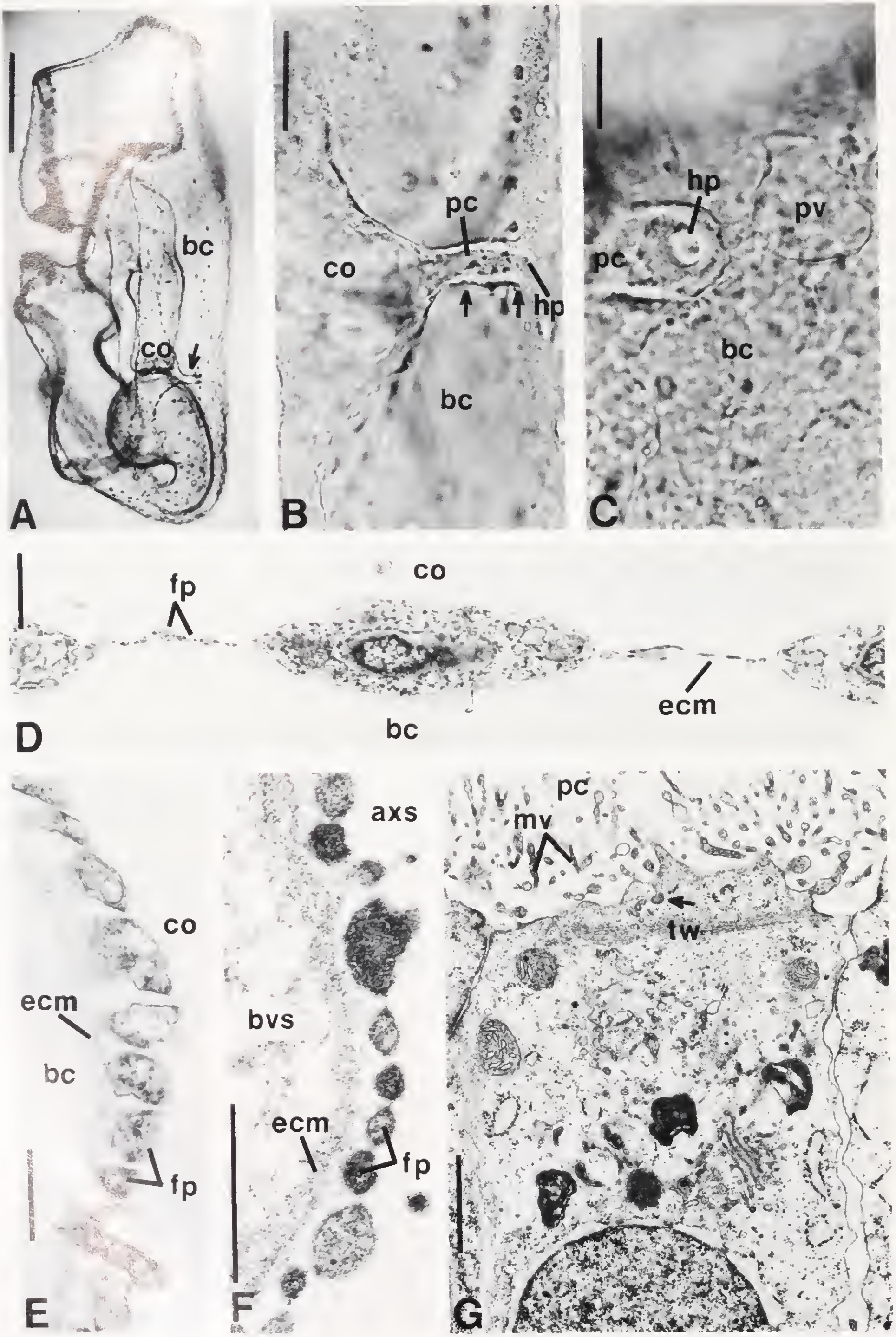
Volumes of internal cavities of both larvae were calculated from digitized tracings (HIPAD digitizer, Houston Instruments, Austin, Texas) of serial cross sections of specimens embedded in Polybed 812 (Polysciences). The computer analysis (Victor 9000, Victor Technologies, Scotts Valley, California) was based on an algorithm for the area of a polygon with vertices (Pearson, 1974).

Morphological data were obtained from living larvae and adults, plastic embedded specimens (Rieger and Ruppert, 1978), and thick sections drawn or photographed on a dissecting (Wild M-5) or compound microscope (Zeiss Photomicroscope I plus drawing tube). Specimens were fixed for thick sectioning and TEM (Philips EM 300) in 2.5% glutaraldehyde in 0.2 *M* Millonig's phosphate buffer for 1 h. Postfixation, after a brief buffer rinse, was in 1.0% OsO_4 in 0.2 *M* buffer for 1 h. After standard alcohol dehydration, the specimens were embedded in Polybed 812.

RESULTS

Development of the complex

Within 72 hours of fertilization at 16°C, the larvae of both species are swimming and possess a ciliated pore canal and dorsal hydropore joining a coelomic cavity to the exterior (Figs. 1A, 2A). A spherical vesicle develops beside the hydropore in the tornaria and near the right coelom in the bipinnaria about 3 weeks into larval life. The vesicle, a coelomic cavity, increases in size as it migrates through the blastocoelic jelly to graft, along two of its sides, near the junction of the pore canal and its coelom (Figs. 1C, 2C). An open cavity, continuous with the blastocoel, remains between one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wall of the vesicle and the pore canal coelom (Fig. 2C). Once in place, the vesicle undergoes a contraction every 5–10 seconds.

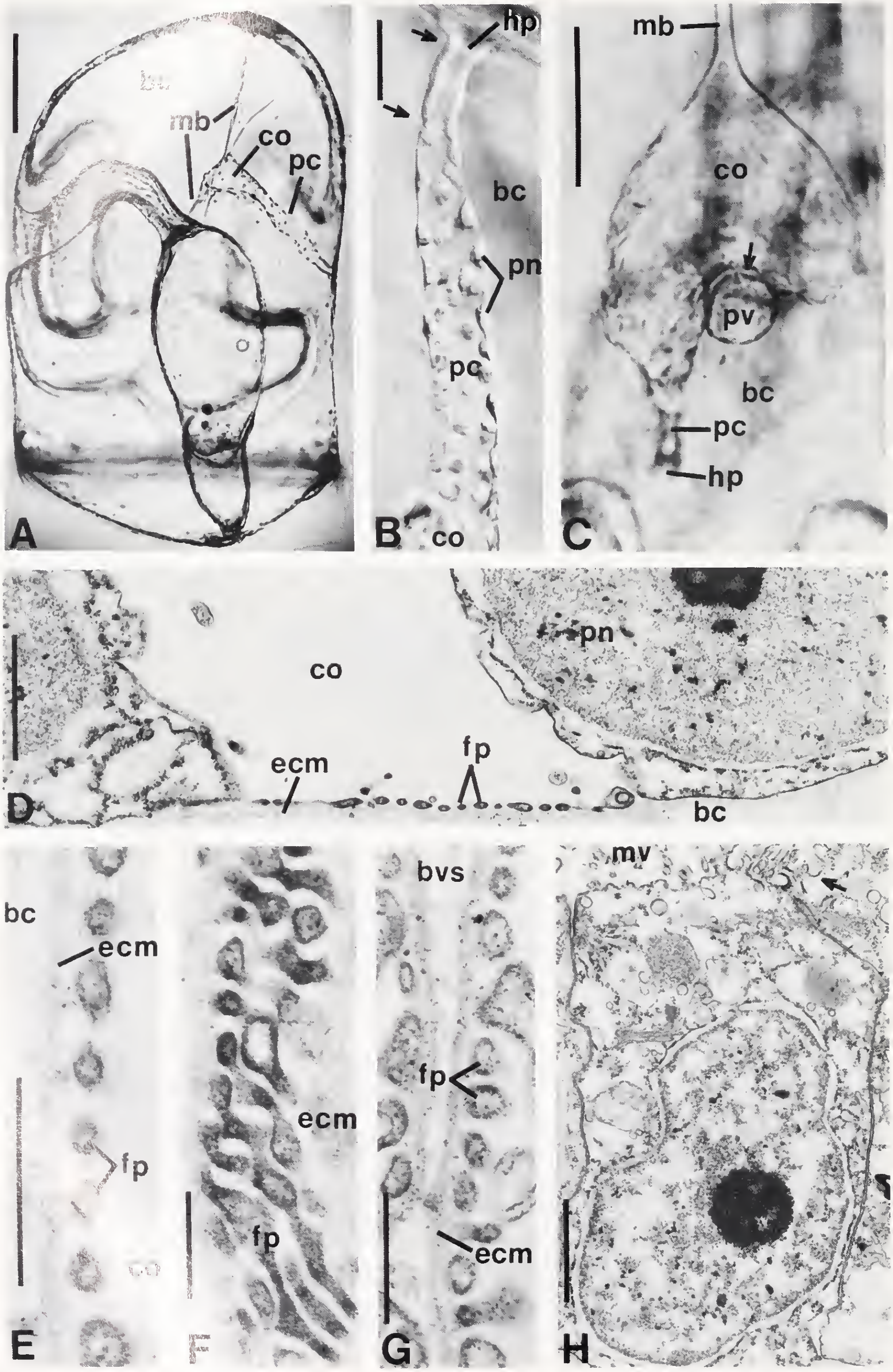
Fluid transport

Direct microscopic examination and cinemicrography revealed a continuous, ciliary-driven efflux of particles from the coelom and pore canal at the hydropore (Fig. 3). All cells forming the pore canal are ciliated as are most of the peritoneal cells lining the coelomic cavities. Table I contains morphometric data for the two larvae. The calculated average particle velocity was 46 $\mu m \cdot s^{-1}$ (±6.25 SE, n = 4, tornaria) and 88 $\mu m \cdot s^{-1}$ (±8.9 SE, n = 2, bipinnaria) in the ectodermal portion of the pore canal. From the dimensions of these cylindrical canals, calculations were made of the fluid volume cleared at the hydropore. Assuming a parabolic velocity profile across the pore canal, the volume cleared is 5.4 $nl \cdot h^{-1}$ by the tornaria and 27 $nl \cdot h^{-1}$ by the bipinnaria. At the calculated rate of discharge through the hydropore, the tornaria will replace its coelomic volume once every 13 min and the bipinnaria every 56 min. Because the coeloms do not collapse with the constant hydroporic efflux, fluid must be recovered from the blastocoel across the coelomic epithelium. Such a flux across the coelomic epithelium raises the possibility of a ciliary-driven ultrafiltration of blastocoelic fluid. Given blastocoelic volumes of 0.27 μl and 0.14 μl in the tornaria and bipinnaria, the entire blastocoelic volume could be cleared in 50 h and 5 h, respectively. The percent body water discharged per hour is approximately 2% for the tornaria and 14% for the bipinnaria.

Ultrastructure of the filtration surface

Transmission electron microscopy of the coelomic lining of the anterior coelom and mesodermal part of the pore canal in the early tornaria reveals that the entire lining is composed of podocytes lying on a thin basal lamina except for a few myoepithelial cells forming the apical muscle band (Fig. 2B, D, E, F). In the definitive tornaria, the podocytic lining is restricted to the region of contact between the coelom and pulsatile vesicle, and along the proximal portion of the pore canal (Fig. 2C). The remainder of the lining differentiates as myoepithelial cells that will contribute to the musculature of the adult proboscis. In the early and definitive bipinnaria, portions of the left coelom at the level of the hydropore are also lined by podocytes, especially along the medial and mediodorsal walls (Fig. 1D, E). Other coelomic regions have not been surveyed.

FIGURE 1. Hydropore-pore canal complex of the bipinnaria larva of *Asterias forbesi* (Asteroidea). (A) Left lateral view of living early larva showing left coelom (co), blastocoel (bc), and pore canal (arrow; bar = 200 μm). (B) Lateral view of pore canal (pc) traversing blastocoel (bc) from left coelom (co) and opening dorsally at the hydropore (hp). Arrows define limit of ectodermal part of canal (bar = 50 μm). (C) Dorsal view of pore canal (pc), hydropore (hp), and pulsatile vesicle (pv) in the blastocoel (bc). Complex is not yet fully organized. Top of micrograph is anterior (bar = 30 μm). (D) TEM of peritoneal podocytes from medial wall of left coelom at the level of the pore canal. The basal lamina (ecm) is the only continuous barrier between the blastocoelic (bc) and coelomic (co) compartments (fp, foot processes of podocytes; bar = 1.0 μm). (E) TEM of podocyte foot processes (fp) from same anatomical region as in D (bc, blastocoel; co, left coelom; ecm, basal lamina; bar = 0.5 μm). (F) TEM of a portion of an axial gland blood vessel (bvs) of a juvenile *Asterias* (see Fig. 4; axs, axial sinus coelom; ecm, basal lamina; fp, foot processes of peritoneal podocytes; bar = 0.5 μm). (G) TEM of columnar cell from ectodermal part of larval pore canal (pc). Each cell bears a single cilium (not shown) projecting into the pore canal (arrow = coated pit; mv, microvilli; tw, terminal web; bar = 1.0 μm).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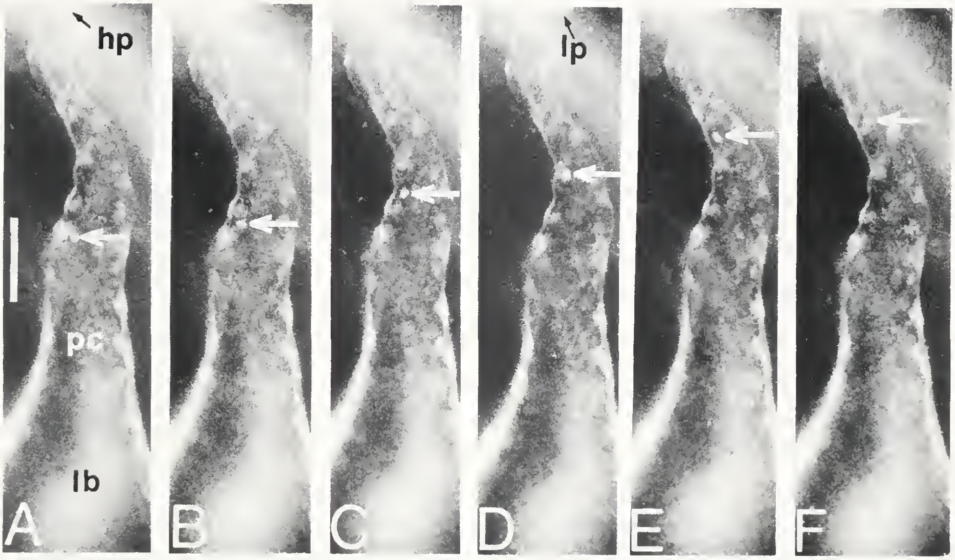

FIGURE 3. Ciliary transport of microbeads in the pore canal of *Schizocardium brasiliense* (Enteropneusta). (A–F) Cinemicrographs of a clump of microbeads in the mesodermal part of the pore canal (arrows; 0.25 s interval between frames). Velocity measurements for the calculations in the text were obtained only for the ectodermal part of the canal (hp, hydropore; lb, latex microbeads in anterior coelom; lp, plume of microbeads expelled at hydropore; pc, pore canal; bar = 50 μm).

The ectodermally derived part of the pore canal adjoining the hydropore in both species is organized as a cuboidal to columnar, ciliated epithelium (Figs. 1G, 2H). The microvillar density and vesicular content of these cells are significantly higher than those associated with the adjacent, overlying, squamous larval epidermis.

The metamorphosis of the larval nephridia is described in Figure 4.

DISCUSSION

The results suggest that larval enteropneusts, asteroids, and perhaps larval echinoderms of the remaining four classes, all of which develop pore canal complexes (Hyman, 1955), possess a functional nephridium probably involved in extracellular volume regulation. The calculated values of 2% and 14% of the body volume cleared

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FIGURE 2. Hydropore-pore canal complex of the tornaria larva of *Schizocardium brasiliense* (Enteropneusta). (A) Left lateral view of living early larva (bc, blastocoel; co, anterior coelom; mb, muscle bands; pc, pore canal; bar = 200 μm). (B) Lateral view of pore canal (pc) traversing blastocoel (bc) and opening dorsally at the hydropore (hp). Arrows indicate ectodermal portion of canal (co, anterior coelom; pn, podocyte nuclei; bar = 40 μm). (C) Dorsal view of definitive complex (anterior is toward top). The spherical pulsatile vesicle (pv) is joined laterally to the wall of the anterior coelom (co) enclosing a small cavity (arrow) that is continuous with the blastocoel (bc; hp, hydropore; mb, muscle band; pc, pore canal; bar = 100 μm). (D) TEM of peritoneal podocytes (pn) from proximal pore canal. The podocyte basal lamina (ecm) is the only continuous barrier between the blastocoelic (bc) and coelomic (co) compartments (fp, foot processes; bar = 1.0 μm). (E–F) Transverse and grazing TEM sections of larval podocytes (bars = 0.5 μm). TEM of podocyte foot processes (fp) on small blood vessels (bvs) in the glomerulus of the enteropneust *Saccoglossus kowalevskii* (see Fig. 4; ecm, basal lamina; bar = 0.5 μm). (H) TEM of columnar cell from ectodermal part of larval pore canal. Each cell bears more than one cilium (not shown) projecting into the pore canal (arrow = coated pit; mv, microvilli; bar = 1.0 μm).

TABLE I

Morphometric data for the larvae of Schizocardium brasiliense *and* Asterias forbesi *(volumes in μl)*

Larva	Tornaria	Bipinnaria
Total length (mm)	1.1	1.6
Body vol.	2.9×10^{-1}	1.9×10^{-1}
Blastocoel vol.	2.7×10^{-1}	1.3×10^{-1}
Protocoel vol.	1.2×10^{-3}	—
Pulsatile vesicle vol.	1.1×10^{-4}	1.3×10^{-4}
Enterocoel vol.	—	2.6×10^{-2}
Left mesocoel vol.	2.8×10^{-4}	—
Right mesocoel vol.	4.0×10^{-4}	—
Left metacoel vol.	6.6×10^{-4}	—
Right metacoel vol.	7.2×10^{-4}	—
Gut vol.	1.5×10^{-2}	3.0×10^{-2}
Ectodermal pore canal length (μm)	45	35
Ectoderm pore canal diameter (μm)	9	15

per hour by these larvae fall within or close to the general range of 1% to 10% given by Kirschner (1967) for aquatic animals. It should be noted, however, that the values presented here are rough estimates based on measurements of the few particles that remained in the focal plane of the microscope while in transit along the pore canal. Given the constant hydroporic efflux, fluid recovery by the adjoining coelom must be from the surrounding blastocoel across the layer of continuous basal lamina and peritoneal podocytes. The mechanism of fluid recovery across the body wall has not been addressed in this study. We speculate that it is driven by an osmotic gradient (Oglesby, 1981), perhaps established by proteins in the blastocoelic jelly.

A protonephridium can be defined functionally as an excretory organ where filtration occurs on the nephridium (terminal cell) and is driven by cilia whereas, in metanephridial systems, filtration occurs on blood vessels or their analogues and filtration pressure is muscular in origin (Ruppert and Smith, 1985, material in prep.). If invertebrate nephridia are so defined, then the pore canal-hydropore complex of these larvae can be considered as a functional protonephridium with the ciliated cells of the pore canal forming the pump driving filtration. It is possible that, when the pulsatile vesicle is positioned, the system functions as a metanephridium, with the ciliary pump being augmented by a muscular pump, the pulsatile vesicle. Supersedure of a larval protonephridium by a later metanephridium is a common feature of representatives in several phyla, *e.g.*, Annelida (Goodrich, 1945), Phoronida (Emig, 1982), and Mollusca (Brandenburg, 1966).

By following the transformation of the larval nephridium during metamorphosis (summary Fig. 4), it is possible to predict that adult asteroids and enteropneusts also possess discrete, functional nephridia. The heart complex, situated in the proboscis of adult enteropneusts, has the structural components of a metanephridial system, including a capillary bed overlain with podocytes associated with the heart ("glomerulus"; Fig. 2G; Wilke, 1971). Modification of the ultrafiltrate could occur in the proboscis coelom, thus delivering nutrients to the proboscis musculature, a tissue with a poorly developed vascular supply. Some modification may also occur along the proboscis duct before a fluid is discharged by the ciliary excurrent at the proboscis pore (Balser, 1985, material in prep.).

The axial complex in adult asteroids, associated with the madreporite, also has the structure of a metanephridial system. We speculate that vascular fluid in the cap-

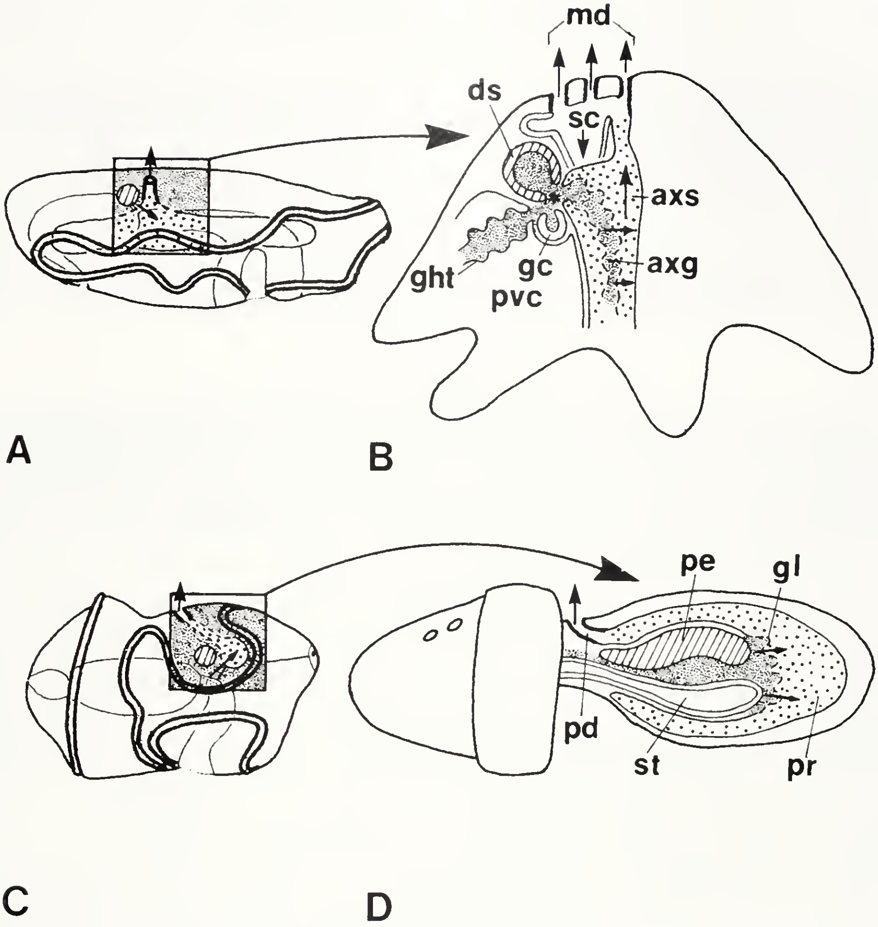

FIGURE 4. Comparison of larval nephridia with adult derivatives. The following conventions apply throughout: small arrows = presumed direction of filtration, larger arrows = observed direction of ciliary beat, cross hatching = pulsatile vesicles in larvae and their derivatives in the adults [madreporic vesicle or dorsal sac (ds) in *Asterias*, pericardium (pe) in *Schizocardium*], dashed lines = coelomic epithelia composed of podocytes, loose stipple = anterior larval coeloms and their adult derivatives (axial sinus (axs) of *Asterias* and proboscis coelom (pr) of *Schizocardium*), fine stipple = blastocoel of larvae and their adult derivative, the blood vascular system, bold lines = larval ectodermal pore canals and adult derivatives [pore canals of the madreporite (md) of *Asterias* and the proboscis duct (pd) of *Schizocardium*]. (A) Lateral view of the bipinnaria of *Asterias* showing nephridial organs. (B) Disproportionately enlarged apical portion of axial complex of adult *Asterias* illustrating anatomical relationships of major components as viewed in vertical section: madreporite (md), madreporic vesicle (ds), stone canal (sc), axial sinus (axs), axial gland (axg), gastric haemal tuft (ght), genital coelom (gc), and perivisceral coelom (pvc). The madreporic vesicle is contractile like its ontogenetic precursor, the pulsatile vesicle. Both the gastric haemal tuft (ght) and axial gland (axg) are tangles of small blood vessels. The gastric haemal tuft spans the pervisceral coelom (pvc) to join the major vessels of the gut while the axial gland vessels join the hyponeural sinus vessels, a portion of the blood vascular system (BVS) and its surrounding coelom that parallels the distribution and extent of the nervous system. The genital coelom (gc) and the BVS it encloses form a ring at the aboral surface of the disc. Vessels from the gut, genital, and peripheral parts of the BVS communicate directly (asterisk) with the vascular cavity ("head process" of axial gland) enclosed by the contractile madreporic vesicle (ds). Pressure for filtration of vascular fluid in the axial gland vessels across the ECM and podocytes of the axial sinus coelom (axs) may be generated by the madreporic vesicle or perhaps by contractions of vessels within the axial gland itself. (C) Lateral view of the tornaria of *Schizocardium* showing nephridial organs. (D) Disproportionately enlarged proboscis organs of adult *Schizocardium* illustrating anatomical relationships of major components: proboscis duct (pd), pericardium (pe), glomerulus (gl), proboscis coelom (pr), and stomochord (st). The pericardium (pe) is contractile like its ontogenetic precursor, the pulsatile vesicle. Pressure for filtration of vascular fluid from the heart and glomerular vessels across the ECM and podocytes of the proboscis coelom may be generated by the pericardium or by contraction of vessels entering the central sinus of the heart.

illary bed of the axial gland is pressure filtered across the layer of peritoneal podocytes (Fig. 1F; Bargmann and von Hehn, 1968) into the axial sinus (a coelomic cavity) where modification could occur. A fluid could be discharged by the ciliary excurrent from the pores of the madreporite.

The recognition of discrete excretory organs in larval hemichordates and echinoderms was prompted by a consideration of a general model for nephridial design and function (Ruppert and Smith, 1985, in prep.). We anticipate that further experimental investigations of other predictions in the model will lead to similar discoveries and an enhanced understanding of renal function and nutrient translocation in animals.

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RESOURCE PARTITIONING BY SAND DOLLARS IN CARBONATE AND SILICEOUS SEDIMENTS: EVIDENCE FROM PODIAL AND PARTICLE DIMENSIONS

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ABSTRACT

The sand dollars, *Leodia sexiesperforata* (Leske) and *Encope michelini* L. Agassiz, have overlapping geographical ranges and may co-occur in mixed flocks. *Leodia* is restricted entirely to biogenic carbonate sediments. *Mellita quinquiesperforata* (Leske), which has a similar geographical range to *Leodia*, occurs only on siliceous terrigenous substrates and the two species never co-exist. *Encope michelini* L. Agassiz occurs on both types of substrate. All three species are podial particle pickers, and use barrel-tipped podia, especially the long type surrounding the geniculate spine fields of the oral surface, for food collection. A typical mellitid of 100 mm diameter can have up to one million barrel-tipped podia. These podia have the same mean diameters in *Leodia* (71.6 ± 5.62 μm) and *Mellita* (71.8 ± 3.59 μm). The diversity of sizes is significantly greater in *Leodia*. The barrel-tipped podia of *E. michelini* are very much larger (104.4 ± 11.1 μm). The substrates inhabited by the three species have approximately 90% of their particles in the 100–400 μm range. Whereas *Mellita* is non-selective in collecting food particles, *Leodia* clearly selects small particles (50–200 μm) and shuns those above 200 μm. *Encope michelini* includes 26% of particles over 200 μm in its food grooves, but does not take those below 100 μm. Differences in feeding behavior thus provide a basis for resource partitioning between these sympatric species. They are discussed in relation to podial dimensions and spination, and compared with feeding behavior in *Mellita quinquiesperforata.*

INTRODUCTION

The feeding activities of sand dollars have been extensively investigated in recent years. It has become clear that many of them rely on oral surface podia for the selection of food material from the substrate. The tiny fibulariid, *Echinocyamus pusillus* (O. F. Müller), picks up diatoms, debris, or sediment particles and conveys them by podia to the mouth where they are chewed or scraped by the lantern teeth (Telford *et al.*, 1983). *Echinarachnius parma* (Lamarck) (Echinarachniidae) ingests sediment material without apparent selection of any particular size range, although diatoms are actively selected (Ellers and Telford, 1984). The lunulate sand dollar, *Mellita quinquiesperforata* (Leske) (Mellitidae), similarly collects sediment particles by means of oral surface podia and fractures them by action of the lantern teeth as they are ingested (Telford *et al.*, 1985). These studies all suggest that sand dollars feed in exactly the way that should be expected of an echinoid, despite their curious shape. These studies have directly challenged the former hypothesis of an aboral sieve mechanism proposed by Goodbody (1960).

In this paper we examine the feeding mechanisms of two more mellitid sand dollars, *Leodia sexiesperforata* (Leske) and *Encope michelini* L. Agassiz, which occur

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in mixed flocks. Our purpose was to determine whether other members of this family use the same podial feeding mechanism and to explore the functional significance of differences in morphology of podia and spines. Two species living together might be expected to show differences in their use of the common food resource. Mooi (1986a, b) has described some differences between the podia of these species. In this paper we examine the relationship between podial dimensions and the size of sediment particles collected during the feeding process. This is the first published description of feeding in any species of *Encope* and the first re-examination of *Leodia sexiesperforata* since Goodbody (1960) proposed the sieve mechanism for that species. A direct comparison will be made between these two species, which live on biogenic carbonate sediments, and *Mellita quinquiesperforata,* which lives on terrigenous siliceous sediments.

MATERIALS AND METHODS

Specimens of *L. sexiesperforata* and *E. michelini,* 50–110 mm in length, were collected in shallow water (3–15 m) off Long Key, Florida, during July, 1984, and maintained in running seawater with natural substrate in the laboratory. Observations of feeding and ciliary currents were made on live specimens using methods described elsewhere (Telford *et al.,* 1985). Several specimens were fixed in the field, in 10% formalin buffered in seawater, for analysis of gut and food groove contents. Six sediment samples of approximately 500 ml were collected from the surface (top 20 mm) in different places among the sand dollar flock. These were fixed in 10% buffered formalin to preserve living organisms and organic material. Larger samples were collected from time to time and kept fresh for use in holding tanks and for feeding observations. Additional specimens of *L. sexiesperforata* and substrate samples obtained from Eleuthera, Bahamas, in February, 1983, were preserved in the same way. Measurements of podia and distribution of podial pores in specimens of *E. michelini* and *L. sexiesperforata* collected at Torch Key, Florida (1982) were compared with specimens of *Mellita quinquiesperforata,* collected at Atlantic Beach, North Carolina (1982).

Specimens of the three species, from personal collections and those of the United States National Museum (USNM), were examined as follows (USNM catalog numbers in parentheses):

> *Leodia sexiesperforata:* personal collections from Bahamas, Barbados, Florida Keys and Panama; USNM collections from Bermuda (E14495), Bahamas (E14892, E9009, #32651), Cuba (E10384), Dominican Republic (E14559), Puerto Rico (#19656), St. Thomas (E1183), St. Kitts (#7000), Windward Islands (E14560), Belize (#18932), Panama (#14579), Colombia (E14561), and Brazil (#5388).
>
> *Mellita quinquiesperforata:* personal collections from North and South Carolina, Georgia, Atlantic and Gulf coasts of Florida; USNM collections from Virginia (#4980), Alabama (E15912, E15914, E15918, #25416-22), Louisiana (E6797), Texas (E6581-2, E5350), Panama (E14584), Colombia (E8091-2), Trinidad (E14062), Puerto Rico (E6608-11), and Brazil (E17195).
>
> *Encope michelini:* personal collections from Long Key, Pigeon Key and Torch Key, Florida; USNM collections from South Carolina (E30005), Georgia (E29843-6), Gulf coast of Florida (#2185), Gulf of Mexico (E26711, E26714) and Brazil (E26706).

Podia (Mooi, 1986a, b) and spines (Telford *et al.,* 1985) were classified and the distribution of different types on the sand dollars was mapped. Isolated spines were measured by ocular micrometer; inter-spine distances were similarly estimated from live and freshly killed specimens under a binocular microscope. Distribution of cilia on different spine types was examined by light microscopy of isolated spines.

Tip diameters of barrel-tipped podia were measured by eye-piece micrometer. Tissue was scraped away from the oral surface in ambulacra I, II, and III from four different specimens of each species, and mounted on a microscope slide. Diameters of 20 podia from each ambulacrum were determined. The data were pooled for each species and means and standard deviations calculated for the combined 240 measurements. Large specimens were chosen so that differences between species would not be obscured by size differences. Specimens of *E. michelini* ranged in size from 93.3 × 94.0 to 104.0 × 105.8 mm; *L. sexiesperforata* from 91.1 × 90.8 to 101.6 × 108.0 mm and *M. quinquiesperforata* from 92.8 × 97.8 up to 98.0 × 99.6 mm. Numbers of pores within the geniculate spine fields of these specimens were determined by cutting out small pieces of test and dissolving soft tissues in 5.25% sodium hypochlorite (commercial strength bleach). Cleaned pieces were dry-mounted on a slide and examined with transmitted light. The total number of podial pores in each of five fields of view in each ambulacrum (I, II, and III) was determined. The data were pooled for each specimen and the means calculated as number of podial pores per square millimeter of geniculate spine field. Estimates of total oral surface area and of the different spine fields were made by cutting out photographic reproductions of the sand dollars and weighing the individual areas.

Analyses of natural substrate, food groove material, and gut contents followed the methods developed by Ellers and Telford (1984) and by Telford *et al.*, (1985). Very small samples of substrate were strewn on glass microscope slides. All particles within several fields of view were drawn in outline by camera lucida after which length and width of at least 1000 from each sample were measured. Material from the food grooves was treated in the same way, but the sample sizes were somewhat smaller. Sediment grains were assigned to size classes 0–24, 25–49, 50–99, 100–199, 200–399, 400–799, and >800 μm and size-frequency histograms were constructed. The ϕ units used by geologists were not used because our interest centered on the numbers and size of sand grains and diameters of podial tips, not on sieve analyses of mass. However, our sediment analysis program also calculated the size-frequency distributions according to size classes for a standard sieve series, for purposes of comparison with other published data. Mean, standard deviation, and elongation (width/length) was calculated and the degree of angularity of the grains was estimated (Leeder, 1982). The significance of differences between particle size-frequency distributions of individual substrate samples and food groove samples was tested by Chi-square analysis, and between means by the t-statistic. All statistical procedures followed Sokal and Rolf (1981). Acid soluble carbonate was determined gravimetrically following digestion in HCl or EDTA.

RESULTS

Ciliary currents among the spines of *L. sexiesperforata* and *E. michelini* are similar to those already described for *M. quinquiesperforata* (Telford *et al.*, 1985). On the aboral surface two quite distinct patterns of flow occur. Within the petaloids, there is a central area of centrifugal flow. Between the respiratory podia, flow is directed from the center of the petaloid (ambulacrum) towards the outside (interambulacrum). Internally, coelomic fluid and cells flow from the outer to the inner pore of each podium, exactly counter to the external current. Around the petaloids, in the interambulacra, the respiratory flow is entrained in a general centrifugal current. Centrifugal flow appears to be diverted close to the lunules, so that their walls are washed by a downward flow within the depth of the spine field. It is easy to exaggerate the current towards the lunules. Flow is usually visualized by means of suspended particles which may occur naturally (diatoms, *etc.*), or be introduced (*Artemia* eggs, black ink, etc.). Particles never describe a straight centrifugal trajectory. The spines are placed somewhat like the pins in a pinball machine. Suspended particles may be carried around a spine, or be driven to the right or left. Thus a group of particles will tend to fan out in the centrifugal flow and, inevitably, some will be carried passively towards the lunules. As in *M. quinquiesperforata*, most particles reaching the ambitus or passing via the lunules, are deposited into the sediment. There is a complex pattern of flow across the oral surface, which must be visualized in three dimensions. In the centers of the locomotory spine fields and the pressure drainage channels (Fig. 1) flow is centripetal but strongly divergent towards the margins of those zones, where it is deflected downwards. At the peristome small centripetal components remain and these unite in a downward flow beneath the mouth. There are some small differences in the flow patterns of *M. quinquiesperforata* and other species treated in this paper and they can be best attributed to differences in the relative sizes of the spine fields. Estimates of

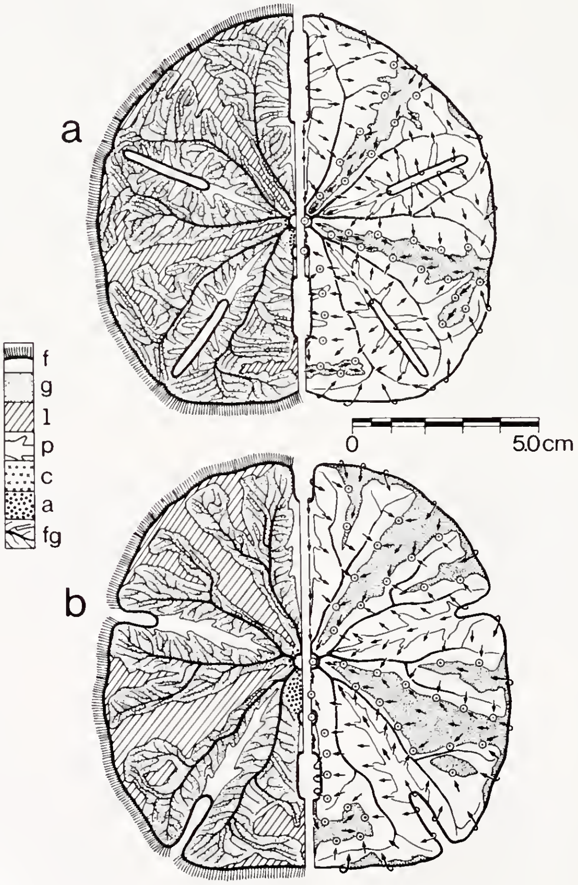

FIGURE 1. Distribution of spine types (left half) and ciliary currents (right half) on (a) *Leodia sexiesperforata* and (b) *Encope michelini*. For left half: f, fringe spines; g, geniculate spines; p, pressure drainage channel (pdc) spines; c, circum-oral spines; a, anal spines; fg, food grooves. For right half: arrows indicate direction of ciliary current flow; circles with central dot show convergence of currents with resulting downward flow to the substrate; locomotory areas stippled.

particle velocities in the ciliary currents were alike in all three species, ranging from 0.50 to 0.95 $mm \cdot s^{-1}$.

In both *E. michelini* and *L. sexiesperforata,* aboral spination is very similar to that of *M. quinquiesperforata* (Telford *et al.,* 1985), but those of *Leodia* are more slender, those of *E. michelini* generally larger and more robust. The spines have the same orientations with respect to body axis, anterior to posterior size gradations and ratios of miliary to club-shaped spines. Spines of the oral surface are equally diversified into locomotory, geniculate, pdc spines, but in *Leodia* and *Encope,* the locomotory areas are relatively smaller and the geniculate areas correspondingly larger. In *M. quinquiesperforata* the locomotory spines occupy more than 30% of the aboral surface area, whereas in *E. michelini* and *L. sexiesperforata* they occupy only 25 and 20% of the surface, respectively. Interspine distances for *Leodia* and *Encope* are given in Table I. Locomotory spines in these two species are equally spaced but there are some differences in the spacing of geniculate and other spines. The ciliary currents described above are powered by diametrically opposed bands of cilia which extend for about 100 μm along the shaft of each spine from its base.

Barrel-tipped podia occur between the geniculate spines in all three species. In *E. michelini* the tip diameter is 104.4 ± 11.08 μm (n = 240); in *L. sexiesperforata* it is 71.6 ± 5.62 μm (n = 240) and in *M. quinquiesperforata* it is almost identical (71.8 ± 3.59), but *Leodia* has a significantly greater range of podial sizes ($P < 0.001$) (Fig. 2). The difference between *E. michelini* and the other two species is statistically significant ($P \ll 0.001$). There are highly significant differences between the densities of podial pores in these three species ($P \ll 0.001$) (Table II). Geniculate spine fields occupy approximately 75% of the total oral surface in *Leodia,* about 70% in *E. michelini,* and 65% in *Mellita.* Pressure drainage channels represent only about 5% of oral surface area in all three species.

Leodia sexiesperforata and *E. michelini* were collected from mixed flocks in shallow water, 5–10 m depth. They were always found on biogenic, carbonate sediments con-

TABLE I

Interspine spacing (μm) in Leodia sexiesperforata *and* Encope michelini

	L. sexiesperforata		*E. michelini*	
Spine types	Space	±S.D.	Space	±S.D.
Locomotory-locomotory	303.0	30.2	297.8	38.2
Locomotory-miliary	190.4	26.5	180.0	35.7
Pdc-pdc	498.5	57.8	448.2	70.0
Pdc-miliary	214.8	33.8	240.7	63.3
Geniculate-geniculate	181.5	22.0	223.7	46.9
Anterior fringe-fringe	277.0	37.2	317.0	50.5
Posterior fringe-fringe	186.7	31.1	281.5	43.8
Anterior club-club	286.7	41.5	263.0	37.8
Anterior club-miliary	134.8	30.5	144.4	19.3
Anterior miliary-miliary	79.3	20.4	129.6	17.7
Posterior club-club	321.1	35.1	201.5	37.5
Posterior club-miliary	80.5	13.8	88.2	20.2
Posterior miliary-miliary	62.2	14.0	58.5	14.8
Anal lunule marginals	233.3	39.3	252.6	35.4
Lunular club-miliary	88.2	18.7	99.3	22.0
Lateral lunule marginals	189.6	33.5	—	—

Spaces given are mean distances between spine shafts immediately above the basal collar. For all measurements, n = 25.

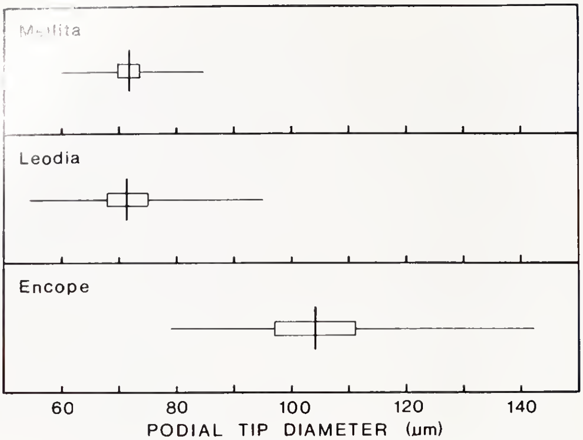

FIGURE 2. Mean (vertical bar), standard deviation (box) and range of sizes (µm) (horizontal line) of short barrel-tipped podia in the geniculate spine fields of *Mellita quinquiesperforata, Leodia sexiesperforata,* and *Encope michelini.*

sisting of fragments of coralline algae (*Halimeda* etc.), shell, and coral debris. Approximately 90% of the particles were in the 100–400 µm range (Fig. 3), with a mean size of 213 ± 68.4 µm. The Eleuthera (Bahamas) sample, where only *Leodia* was found, had a lower mean grain size (158 ± 43.1) due to larger numbers of particles (23%) between 50 and 100 µm. *Mellita quinquiesperforata* occurs on terrigenous, siliceous sediments with a similar percentage of particles in the 100–400 µm range (Fig. 3) and a mean size of 186 ± 63.9. The remaining 10% of the *Mellita* substrate was less than 100 µm. In the biogenic sediment, 6% of the particles exceeded 400 µm. The mean elongation of the rather angular quartz sand grains was 0.70. The biogenic

TABLE II

Density of podial pores per mm² in the geniculate spine fields of Mellita quinquiesperforata, Leodia sexiesperforata, *and* Encope michelini

	Ambulacrum 1	Ambulacrum 2	Ambulacrum 3	Mean	S.D.
Mellita 1	140	168	180		
Mellita 2	136	173	167		
Mellita 3	147	170	178		
Mellita 4	144	168	183		
				163	±16.5
Leodia 1	115	139	143		
Leodia 2	111	136	146		
Leodia 3	120	135	150		
Leodia 4	109	134	140		
				132	±14.1
Encope 1	100	102	103		
Encope 2	98	107	99		
Encope 3	91	104	105		
Encope 4	95	99	102		
				100	±4.4

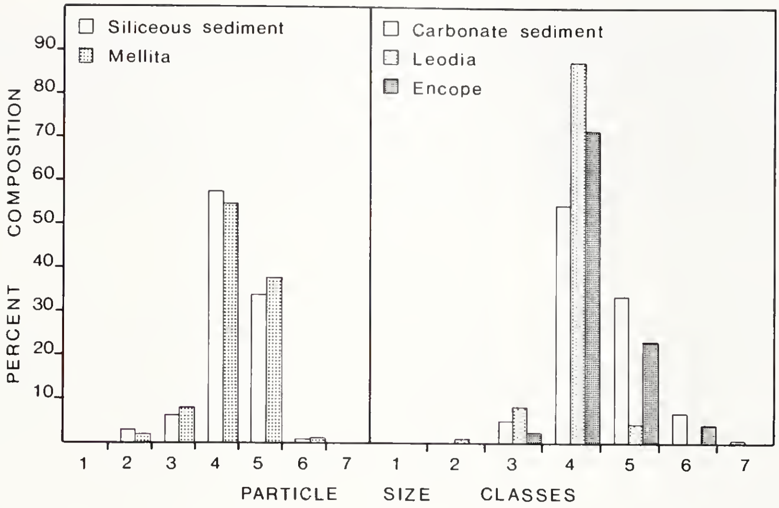

FIGURE 3. Particle size distributions in natural siliceous and carbonate sediments and food grooves of sand dollars inhabiting them. Mean particle size in the siliceous sediment was 186.2 ± 63.91 μm (n = 3109), and in the carbonate sediment 212.9 ± 68.43 μm (n = 3362). Within the food grooves, mean particle sizes were: *Mellita,* 181.7 ± 54.23 μm (n = 2735); *Leodia,* 140.8 ± 34.64 μm (n = 2969); *Encope,* 183.0 ± 88.52 μm (n = 2418).

grains were more rounded, distinctly subangular, with mean elongation of 0.72. Dissolution of small subsamples showed that this sediment was 100% carbonate. However, under the microscope occasional non-carbonate particles were observed. When the biogenic particles were dissolved carefully in 1–2% HCl or in EDTA, delicate organic "ghosts" of the grains became visible.

Leodia and *Encope,* like *Mellita,* are podial particle pickers. During feeding long barrel-tipped podia around the geniculate spine fields pick up individual sand grains which are then passed from podium to podium in an orderly progression towards the food grooves. As the particles are passed along, they can be held beneath the tips of the geniculate spines, in the space above the sediment, or they may pass between the tips of the spines, which are in constant motion during feeding. Upon arrival at the food grooves, the particles are moved centripetally towards the mouth by action of the food groove podia. Adhesion of particles and podia is at least partially due to a sticky glue-like substance (Thomas and Hermans, 1985). As the particles become coated with this material they readily adhere to each other and form cohesive strings in the food grooves. Analysis of the size-frequency distributions of particles in the food grooves (Fig. 3), shows that *Mellita* is non-selective: it collects particles in essentially the same proportions as they occur in the substrate. In the food grooves of *Leodia,* 87% of the particles were in the size range 100–200 μm, and the mean was 141 ± 34.6. Particles in the food grooves of *E. michelini* had a greater mean size (183 ± 88.5 μm). Differences between the mean sizes and the frequency distributions for these two species were statistically significant ($P \ll 0.001$). In both species, food groove material included a slightly higher proportion of forams and diatoms than observed in the native sediment. Particle size-frequency analysis of gut contents from *Leodia* and *E. michelini* was not possible by the methods used here. The contents included a large proportion of well pulverized sand grains, among a few larger particles. Some fragments were large enough to identify as broken forams, diatoms, and shell debris, but most of the material was amorphous and cohesive.

Fine material, such as particles of carmine and black ink (used for flow visualization), were deposited in the sediment around the ambitus. Those particles which were included in oral surface flow were brought to the edges of the locomotory spine fields where they were then deposited by downward currents. Some of this fine material adhered to podia and sand grains. As a consequence, some was included in the food groove material, still clinging to larger particles. We saw no evidence that this fine material was deliberately selected. In fact, most of it was left in the sediment.

DISCUSSION

Leodia sexiesperforata and *Encope michelini* have partially overlapping distributional ranges and often occur together in mixed flocks. *Leodia* ranges from North Carolina to Uruguay, including the Florida Keys, Bahamas, Greater and Lesser Antilles, and the Gulf of Mexico. *Encope michelini* is distributed from North Carolina south to the Florida Keys and throughout the Gulf of Mexico, but not in the Bahamas (Serafy, 1979). *Mellita quinquiesperforata* extends from Massachusetts to Florida, throughout the Gulf, Caribbean, Central and South America to Brazil, as well as all the Antilles (Serafy, 1979). From our own field experience and examination of museum material, it is apparent that *Leodia* occurs only on biogenic sands and that *Mellita* is restricted to terrigenous sediments. It is curious that this very striking feature of distribution has not been remarked upon previously. *Encope michelini* appears to inhabit both sediment types. In our own field studies we have only found this species on the biogenic sediments of the Florida Keys. However, examination of museum specimens from South Carolina, Georgia, elsewhere in Florida, and the Gulf of Mexico, showed mixtures of shell debris and substantial amounts of quartz grains in the food grooves.

The sediment particle sizes at the different collection sites were very similar. The mean particle size of siliceous material of Atlantic Beach (*Mellita*) is remarkably close to previously reported values for Bird Shoal, (180.8 ± 59.74) (Telford *et al.,* 1985) and for Florida (Serafy, 1979) ". . . fine quartz sand with modal grain size of 0.18 mm . . ." They are also quite comparable with the data reported by Weihe and Gray (1968) for their collecting sites in North Carolina. The mixed flocks of *Leodia* and *E. michelini* occur on a substrate incorporating a small but significant number of particles over 400 μm (6%), but otherwise very like the *Mellita* substrate. Many authors, including those already cited, have remarked on the scarcity of fine particles (<50 μm) in sand dollar habitats. The sediment analyses provided by Lane and Lawrence (1982) for a *Mellita* population near Tampa (Florida), showed 92% of the grains in the 125–250 μm size class, 5% in the 62.5–125 class, and the rest smaller than that. Our *Mellita* substrates did include 10% of the particles below 100 μm, but there was a substantial proportion over 250 μm (Fig. 3).

There are small differences in spination between the three species. On the aboral surface, the miliary sacs which fill the spaces between the tips of club spines, preventing the entry of particles during burrowing (Mooi, in press), are largest in *Mellita* and smallest in *E. michelini.* The generally centripetal ciliary currents remove the few small particles which drop through the protective canopy (Mooi, in press; Telford *et al.,* 1985). Interspine spacing on the aboral surface is wider in *Leodia* and *Encope* and the latter has wider spacing between the ambital fringe spines. On the oral surface, spacing between locomotory spines is widest in *Mellita* (460 ± 86 μm) but all other inter-spine distances are somewhat smaller. Within the geniculate spine fields *Mellita* has significantly more barrel-tipped podia per mm^2 (Table II) and *Leodia* likewise has more than *E. michelini.* These are the food gathering podia, which adhere to collected particles. Mean podial diameters reported here for *Mellita* (72 μm), based on large numbers of measurements, are somewhat smaller than those reported by Phelan (1977) (84 μm) and much smaller than our own earlier reported value of 120 μm (Telford *et al.,* 1985) which, in retrospect, appears to represent an extreme and not the typical size of food gathering podia. In fact, these suckered podia in *Mellita* rarely approach 100 μm, even close to the peristome. *Encope michelini* has very much larger podia, none of them as small as the mean sizes for *Mellita* and *Leodia* (Fig. 2).

All three species use the impressively large numbers of podia on the oral surface for the collection and transport of food. *Leodia* has the most extensive geniculate spine areas and we estimate that individuals 100 mm in length have approximately 1×10^6 barrel-tipped podia, of which 150×10^3 are food collecting (long b-t) podia. *Mellita* has some 0.85×10^6 b-t podia (125×10^3 long) and *E. michelini* 0.70×10^6 (100×10^3 long). According to our estimates, the total area of the suckered podial tips in *Mellita* and *Leodia* represents over 40% of the total oral surface area and about 15% of this is the actual food collecting, long barrel-tipped podia. In *E. michelini* the total is even higher, about 60% of the total oral surface area, with a similar proportion of long b-t podia. Ciliary currents do not contribute significantly to the feeding process. As reported earlier (Telford *et al.,* 1985), *Mellita* is non-selective in its feeding. Both *Leodia* and *E. michelini* (Fig. 3) include disproportionately high percentages of the 100–200 μm fraction in their food grooves. *Leodia* also appears to select from the 50–100 μm class and to shun particles greater than 200 μm. *Encope michelini* takes very few particles less than 100 μm and includes significant amounts (26%) above 200 μm. Although one would not expect sand to be a limiting resource for these cohabiting sand dollars, some divergence of feeding and, hence, partitioning of the food resource appears to occur. Hammond (1982) concluded that sympatric holothuroids and echinoids in a similar habitat in Jamaica, did not show any resource partitioning: they all ingested sediment mixtures very similar to the composition of the surrounding sand. Although they are nonselective for particle sizes, Hammond (1983) did find evidence that some holothuroids and echinoids were selective for the organic content of grains. Ellers and Telford (1984) and Telford *et al.* (1985) found that feeding in clypeasteroids could be stimulated by presentation of diatom-enriched material, and both *Leodia* and *E. michelini* included relatively high proportions of forams and diatoms in the food grooves, suggesting that these were being actively selected. Scheibling (1980) found that microphagous feeding in the asteroid *Oreaster reticulatus* was similarly stimulated by the presence of diatoms, suggesting that selection of particles for nutrient content might, indeed, be widespread in deposit feeding echinoderms. The small differences in spination and the larger differences in podial dimensions and distribution, do not supply a ready mechanical explanation for resource partitioning by *Leodia* and *E. michelini.* It might be expected that species collecting larger particles would have wider spaces between the geniculate spines. This does not appear to be borne out by observation. The space between spine tips is constantly changing as the spines move and it is clear that *Mellita,* at least, is able to collect a mean particle size (180 μm) which is greater than the mean stationary inter-spine distance (150 μm). With podia very much like those of *Leodia, Mellita* nonetheless collects food particles more like the size range taken by *E. michelini.* It is possible that spacing between geniculate spines and podial tip dimensions together provide an upper limit to the size of particle handled. We suspect also that the podia are poorly suited to adhesion on particles substantially smaller than themselves and that this sets a lower limit. Certainly, the mean food groove particle sizes are 1.5 to 2.5 times the podial tip dimensions. In a comparison of *E. aberrans* and *E. michelini,* Phelan (1972) speculated that they might prefer different particle size ranges and noted that the dimensions of the food grooves and peristome differed. *Encope michelini* has wider, less distinct food grooves (~750 μm) than either *Leodia* or *Mellita* (~600 μm). Peristome diameters of *Mellita* and *E. michelini* (~3.75 mm) are significantly larger than *Leodia* (2.25 mm), in similar sized individuals. These dimensions are 15–20 times the mean linear dimensions of ingested particles. [illegible] difficult to see how these peristome diameters might influence the size of particles selected as food. We would suggest that *E. michelini* and *Leodia* might tolerate wider sediment size ranges and particle size-frequency distributions. Both have greater podial size diversity than *Mellita*, which might allow them to handle a wider diversity of food particles. We suspect that *Leodia* overlaps the distribution of *E. michelini* only towards the upper and lower extremes of their respective particle tolerances. We can offer no explanation at present for the separation of *Mellita* and *Leodia* on different substrate types, both of which seem to be acceptable to *Encope* species.

When Goodbody (1960) examined stomach contents in *L. sexiesperforata* he was unable to identify much of the material but remarked on the small size of the particles. Until recently this has been one of the mainstays of the sieve hypothesis but Telford *et al.* (1985) concluded that the lantern of *Mellita quinquiesperforata* fragmented sand grains as they were ingested. Carbonate sand grains contain an organic matrix in which the calcite is originally deposited and, in addition, they are extensively penetrated by fine filaments of algae and sponges. It is this organic material which becomes visible under the microscope as an organic "ghost" of the sand grain following slow dissolving. They may thus be a more rewarding nutrient source than siliceous grains bearing only surface organics. The penetration by algae and sponges also makes these grains much more readily breakable by the lantern teeth. For this reason, sand grains ingested by *Leodia* and *E. michelini* are thoroughly pulverized into an unrecognizable paste, whereas the siliceous grains ingested by *Mellita* are broken into smaller but still recognizable granules.

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EFFECTS OF OPIOIDS AND ANTAGONISTS ON THE RATE OF SEA URCHIN SPERM PROGRESSIVE MOTILITY

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ABSTRACT

Opioids exert a profound effect on the motility of sea urchin sperm cells suspended in artificial seawater (ASW). With prolonged exposure to narcotic agents, the rate of progression increased in a time- and dose-dependent manner up to an optimum concentration. Methionine enkephalin acetate (metenkephalin) caused a doubling in the rate of progression while the opiate antagonist, (−)-naloxone caused the sperm cells to increase their rate of forward motion by up to 75% above the control rate. The metenkephalin increase occurred at lower concentrations than the naloxone optimum. In combination, a subthreshold dose of (−)-naloxone, completely abolished the stimulatory effects of metenkephalin. Plus-naloxone, the inactive isomer of (−)-naloxone did not have any effect by itself on sea urchin sperm motility nor did it alter the metenkephalin-induced effect. Even though the magnitude and the nature of the sperm cells' responses to the opioids and antagonists appear somewhat atypical compared to the responses of neurobiological systems, the lack of effect of (+)-naloxone confirms the specificity of the response and is consistent with the presence of opioid receptors in the sperm cell.

INTRODUCTION

Neuroactive agents cause sperm cells to alter their swimming behavior and, in some cases may affect their fertilizing capacity (Atherton *et al.,* 1978; Bavister *et al.,* 1979; Cornett and Meizel, 1978; Nelson, 1978; Sastry *et al.,* 1981).

The sperm cells of mussels, sea urchins, and starfish modulate their behavior when exposed to cholinergic agents (Nelson, 1978). Spermatozoa of vertebrate species from rams (Stewart and Forrester, 1978), bulls (Egbunike, 1982), boars (Sekine, 1951), and rabbits (Bishop *et al.,* 1976) to humans (Zeller and Joel, 1941; Sastry *et al.,* 1981) also tested positively for the presence of acetylcholine, its receptor, acetylcholinesterase, and choline acetyltransferase.

The rate of propulsion of sea urchin spermatozoa varies biphasically both when exposed to acetylcholine and to nicotine (Nelson, 1972). The presence of a nicotinic receptor in the sea urchin sperm was confirmed when it was shown that both d-tubocurarine (Nelson, 1973) and α-bungarotoxin (Nelson, 1976) inhibited their motility. Low concentrations of decamethonium, which interacts with the cholinergic receptor at neural synapses and neuromuscular junctions, caused a slight transitory increase in the rate of motile progression but depressed the motility at slightly higher concentrations (Nelson, 1973).

Cholinergic agents have been postulated to affect the transmembrane and intracellular transport of calcium into and within the sperm cell (Nelson, 1978). This

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interdependence resembles the acetylcholine-calcium relationship in brain cell synaptosomes demonstrated by the inhibition of the release of acetylcholine due to the blocking of calcium uptake when the synaptosomes are incubated in the presence of morphine (Sanfacon *et al.*, 1977). The action of β-endorphin on rat brain synaptosomes was even more potent (Guerrro-Munoz *et al.*, 1979).

It was found that after a delay period, morphine-treated spermatozoa of the Mediterranean sea urchins, *Arbacia lixula, Paracentrotus lividus,* and *Sphaerechinus granularis,* appeared to swim more rapidly and for longer periods than untreated control cells under microscopic examination (Cariello and Nelson, 1984). The present experiment was designed to test whether the opioid effect on sperm cell behavior could be interpreted in terms of the effects of the narcotics on the brain synaptosome model.

MATERIALS AND METHODS

To quantify the effects of the treatment, spermatozoa of the purple sea urchin, *Arbacia punctulata,* (the sea urchins, kept in running seawater aquaria were replenished several times weekly as needed during June, July and August) were aligned and oriented under gentle centrifugation in the horizontal rotor of an IEC Model CL centrifuge. Male sea urchins were induced to spawn by intracoelomic injection of 0.5 *M* KCl. One-tenth ml of the concentrated sperm cells was diluted in 20 ml of artificial seawater (ASW) (The turbidity of the suspension was adjusted to an optical density of 0.5–0.7, equivalent to 7–9 $\times$ 10^6 cells/ml.) One liter of ASW contains: NaCl, 24.72 g; KCl, 0.67 g; $CaCl_2 \cdot 2H_2O$, 1.36 g; $MgCl_2 \cdot 6H_2O$, 4.66 g; $MgSO_4 \cdot 7H_2O$, 6.29 g; and $NaHCO_3$, 0.18 g. Two ml of the sperm cell suspension were pipetted into each of 6 round, 1-cm diameter cuvettes. The control cuvette contained 0.2 ml of ASW; the second cuvette contained 0.2 ml of 10% formaldehyde; the remaining 4 cuvettes contained 0.2 ml of a given concentration of the test reagent. Each cuvette was inverted 2× to disperse the contents. The suspensions were preincubated for 0, 5, 10, and 15 minute periods at room temperature (22–25°C) and the sperm cells were aligned at 120 $\times$ *g* for 4 min. This centrifugal force orients the cells while displacing the formaldehyde-killed and non-motile cells only minimally or not at all (Nelson, 1972). Treated cells then swim more rapidly or more slowly than the control cells toward the bottom of the cuvettes and the optical densities (O.D.) at 540 nm are measured in a Bausch and Lomb model 340 spectrophotometer, before and after orientation. Readings were corrected for any displacement of the killed cells and forward motility was expressed as percent of the change in O.D. of the control suspension normalized to 100 (Nelson, 1972). The effect of each concentration of the opioid or antagonist was determined on at least six different samples for each incubation period. The sperm cells were exposed to methionine enkephalin acetate (Sigma) over a concentration range of 0.0016 to 1.23 m*M* and to (−)-naloxone-HCl (Endo) over a range of 0.0016 to 4.87 m*M* (Figs. 1, 2). The effect of competition between the opioid and the antagonist was tested at a fixed concentration of 0.005 m*M* naloxone over the entire concentration series of metenkephalin (Fig. 3). Plus-naloxone, (kindly supplied by NIDA), the enantiomer of (−)-naloxone, which presumably has much less ability to interact with the opiate receptor (Iijima *et al.*, 1978), was diluted to concentrations of 0.0016 m*M* to 1.09 m*M* in ASW. Plus-naloxone was tested both alone and in combination with the metenkephalin for the sea urchin sperm motility responses (Fig. 4).

RESULTS

The *Arbacia* sperm responded maximally to metenkephalin at a concentration between 0.1 and 0.2 millimolar whether or not the cells had been preincubated in the

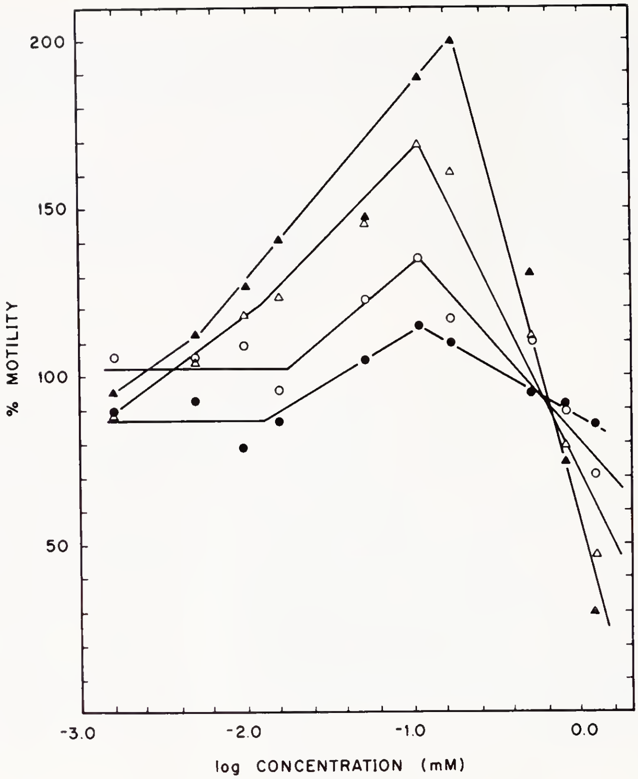

FIGURE 1. Motility of *Arbacia* sperm suspended in ASW containing 0.0016 to 1.23 m*M* metenkephalin. Abscissa: log conc. in m*M*. Ordinate: percent change in motility (control taken as 100%) after orientation at 120 × *g*. Symbols represent periods of preincubation before orientation. Solid circles, 0 min; open circles, 5 min; open triangles, 10 min; solid triangles, 15 min.

opioid before centrifugal orientation. While the brief exposure of the "zero-time" cells during centrifugation elicited only about a 15% increase in progressive rate at that concentration, less than 5 micromoles per liter appeared to suffice as a threshold concentration for the metenkephalin preincubated for 15 minutes before alignment. Concentrations of the opioids greater than 1 mM/l ASW were inhibitory (Fig. 1).

The response to the opiate antagonist, (−)-naloxone, began to develop at about 25 micromolar while maximal stimulation occurred at 0.5 millimolar and lesser increases in forward motility appeared at slightly higher concentrations, inhibition occurring at 2 millimolar and above (Fig. 2). Metenkephalin caused a doubling at its optimum while naloxone stimulated the sperm to a 75% increase over the control rate at its optimum when the sperm cells were preincubated for 15 minutes. Nearly complete inhibition with 15 minutes exposure occurred at about 1 millimolar metenkephalin but it required about 5 times that amount of (−)-naloxone to cause nearly complete cessation of movement.

Since both metenkephalin and (anomalously), (−)-naloxone appeared to exert delayed stimulatory effects on the spermatozoa to somewhat different though overlapping concentrations, it was important to test for interactive effects of the opioid and the so-called antagonist. This is illustrated in Figure 3, a composite of the graphs

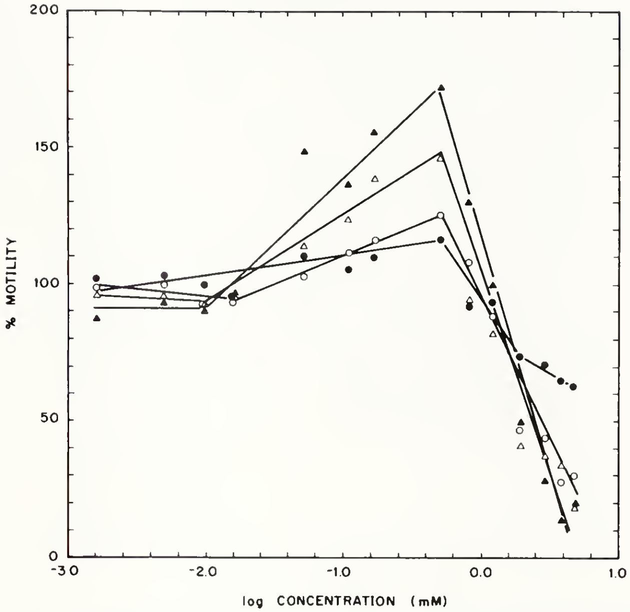

FIGURE 2. Motility of *Arbacia* sperm suspended in ASW containing 0.0016 to 4.87 mM (−)-naloxone-HCl. Abscissa: log conc. in m*M*. Ordinate: percent change in motility after 4 min orientation at 120 × *g*. Symbols, as in Figure 1.

of the results of the 15-minute preincubation periods for metenkephalin, open circles; (−)-naloxone, solid circles; and the mixture of the two compounds, open squares. In the combined drug study, the sperm cells were incubated at room temperature in suspensions containing about 1/5 the minimally effective concentration of (−)-naloxone, 5 μmole/l, over the whole concentration range of metenkephalin assayed (from 0.0016 to 1.23 millimolar). Alone, metenkephalin depressed motility below the control rate at about 0.8 m*M* and increased the motile rate by about 15% at 7 μ*M*. The subliminal concentration, 5 μ*M*, of (−)-naloxone completely blocked all stimulatory responses to the metenkephelin, although the rate of decline in motility induced by supraoptimal concentrations of either the opioid or antagonist alone was less pronounced in the combined drug medium (Fig. 3).

Since the (−)-naloxone exhibited atypical pharmacological action in the sea urchin sperm system, the inactive enantiomer, (+)-naloxone, was tested for its effects in the motility assay. Figure 4 shows the sperm cell responses to this drug, both alone and in combination with metenkephalin. By itself, (+)-naloxone (triangles) had no effect on motility at concentrations between micromolar and millimolar. Similarly, the increased motility due to metenkephalin (circles) was unaffected when the incubation medium contained 5 micromolar (+)-naloxone along with the metenkephalin (x's), in contrast to the reversing effect of 5 micromolar (−)-naloxone on metenkephalin shown in Figure 3. The data in Figure 4 depict the effects after a 15 min incubation. Similarly, the curve for the (−)-naloxone alone (squares) measured concurrently shows comparable characteristics with the previous experiment in Figure 3 (The data for Figs. 1, 2, and 3 were collected in July, 1984, those for Fig. 4 in July, 1985.)

In preliminary experiments morphine sulfate exerted a depressant effect on *Sphaerechinus* sperm during brief exposure, but following prolonged incubation, up to 45

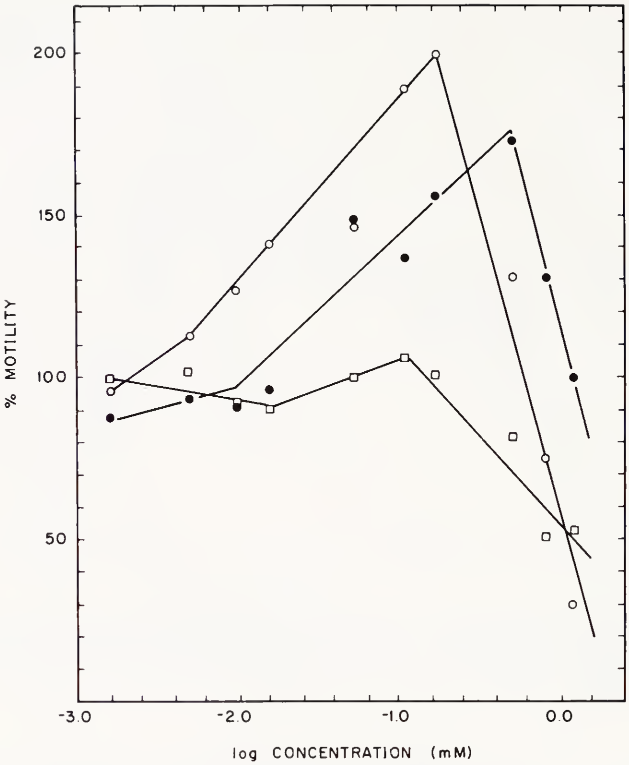

FIGURE 3. Drug interaction effect on *Arbacia* sperm. Incubation mixture contains (−)-naloxone at a fixed concentration of 0.005 m*M* and varying amount of metenkephalin. Ordinate: percent change in motility after 15 min incubation. Abscissa: log conc. in m*M*. Open squares 0.005 m*M* (−)-naloxone and 0.0016 to 4.87 m*M* metenkephalin; open circles, metenkephalin alone, 0.0016 m*M* to 1.23 m*M*; solid circles, (−)-naloxone alone 0.0016 to 4.87 m*M*. Note that 0.005 m*M* (−)-naloxone completely blocks stimulation due to metenkephalin.

minutes, the rate of sperm progression increased to 70% above that of the controls (Cariello and Nelson, 1984).

DISCUSSION

Responsiveness to the action of the pharmacological agents by sperm cells is quantitatively demonstrated in terms of changes in their rate (or pattern) of motile progression. We have here presented evidence that opioids and opiate antagonists elicit both time- and dose-dependent alterations in *Arbacia* sperm swimming rate. But it is quite evident that suspensions of living sperm cells of marine invertebrates, while sharing some physiological properties with the sperm of vertebrates yet exhibit characteristic behavioral responses that differ both qualitatively and quantitatively from other excitable tissues.

Intact sea urchin sperm cells respond to narcotics and antagonists. These experiments were undertaken following our preliminary observations that morphine had a pronounced effect in the sperm cells of the Mediterranean sea urchin, *Sphaerechinus granularis* (Cariello and Nelson, 1984). Quantitative determinations on the motility

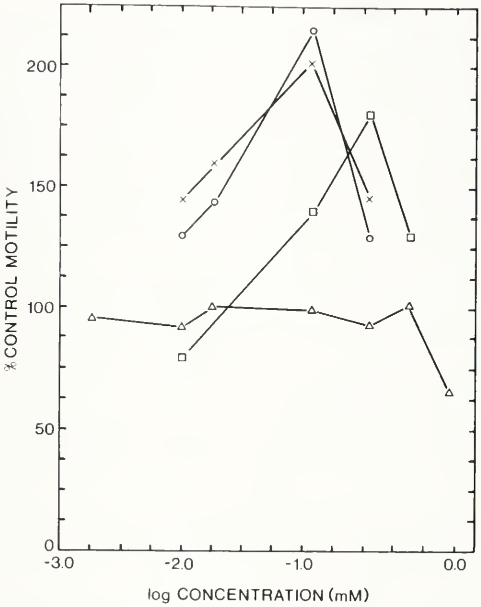

FIGURE 4. Plus-naloxone and *Arbacia* sperm. Abscissa: log conc. in m*M*. Ordinate: percent change in motility after 15-min incubation in ASW containing (+)-naloxone alone (open triangles); (−)-naloxone alone (open squares); metenkephalin alone (open circles); and metenkephalin and (+)-naloxone 0.005 m*M* (× - - - ×).

of sperm cells of *Arbacia punctulata* suspended in ASW showed that metenkephalin acetate caused a biphasic dose-dependent response (Fig. 1). Metenkephalin doubled the rate of progression measured after a 15-minute preincubation period, with lesser degrees of motility enhancement after shorter exposure. At concentrations greater than optimum, metenkephalin depressed motility more acutely with increasing exposure time. This aspect is brought into focus in the drug-interaction depicted in Figure 3 in which the subthreshold concentration of (−)-naloxone, having reversed metenkephalin's stimulatory effect at suboptimal amounts, becomes overridden allowing motility to decline as the metenkephalin was applied in supraoptimal doses. Under these conditions (−)-naloxone fulfills its putative role as a competitive opiate antagonist even though by itself it acts atypically in the sea urchin sperm system. Usually (−)-naloxone does not exhibit agonistic effects and so is considered to be a relatively pure antagonist which may be taken as an indicator for opioid receptor-mediated physiological processes and, biochemically, in ligand-displacement assays of naturally occurring morphine-like factors. While (−)-naloxone is almost devoid of agonistic effects in whole animal or organ-receptor assay, nevertheless it appears by virtue of the blocking of metenkephalin stimulation to be of the class of substances referred to either as partial agonists or agonist-antagonists in the context of the sea urchin sperm system.

The specificity of the sperm opioid receptor appears to be attested not only by (−)-naloxone's blocking of metenkephalin's agonist action, but further confirmed by the inability of (+)-naloxone to influence metenkephalin's stimulation as well as by the fact that (+)-naloxone itself is without discernible effect on *Arbacia* sperm motility at concentrations below 1 millimolar. According to Iijima *et al.* (1978), who synthesized it from thebaine, the (+)-naloxone had no more than 1/1000 to 1/10,000 the pharmacological action of (−)-naloxone and can therefore "serve to test the stereospecificity of the biochemical and pharmacological actions of (−)-naloxone." These investigators tested the (+)-naloxone on three bioassay systems including the rat brain membrane receptor binding assay, guinea pig ileum, and the reversal of morphine inhibition of the adenylate cyclase activity of a neuroblastoma × glioma hybrid.

In the *Arbacia* sperm motility assay the threshold for metenkephalin stimulation fell in the micromolar range, that due to (−)-naloxone commenced at an order of magnitude higher, while above one millimolar, (+)-naloxone showed only a slightly depressant effect but did not reverse the metenkephalin effect at all. By these criteria, the *Arbacia* sperm may be considered to be endowed with opiate receptors that may bind endogenous opioids and also the opiate antagonist (−)-naloxone. Sastry and coworkers (1982) have extracted enkephalin-like and substance P-like compounds from human, rat, and bull spermatozoa, and from human seminal plasma and from rat male accessory glands which they detected by radioimmunoassay. The seminal plasma contained higher levels of met- and leu-enkephalins than did the sperm, while the distribution of substance P in these tissues was the reverse. These investigators point to the role of the opioid peptides in regulation of acetylcholine-induced Ca^{2+} fluxes which are essential for sperm motility and the acrosome reaction.

Immunoreactive β-endorphin-like material was detected in Leydig cells, epidydymal epithelia, seminal vesicles, and vas deferens of rat, mouse, guinea pig, hamster, and rabbit (Shu-Dong *et al.*, 1982). Fravioli *et al.* (1984) investigated the possible role of β-endorphin, met-enkephalin, and calcitonin in human sperm motility regulation. In their study, they report that metenkephalin does not affect the motility at concentrations between nanomolar and millimolar, although β-endorphin starts to depress motility at about 0.5 micromolar. On the other hand, calcitonin, in seminal fluid at about three times its concentration in peripheral blood plasma, markedly depresses human sperm motility in the nanomolar range and completely inhibits movement at micromolar concentration. It is not clear at this time why (−)-naloxone behaves anomalously in stimulating *Arbacia* sperm, nor why metenkephalin's stimulatory effect on the sea urchin sperm should differ from its reported lack of effect on human sperm.

Similarly unanticipated results with opioids have been reported by Zagon and McLaughlin (1983, 1986). Heroin inhibition of the growth of transplanted neuroblastoma in mice was blocked by concomitant administration of naloxone. Paradoxically, when naloxone was tested alone at concentrations sufficient to interact with opiate receptors, this opiate antagonist was extremely effective in prevention or retardation of the tumor growth. According to these investigators, the antagonist naltrexone can promote tumorigenesis at a dose that prevents the analgesic action of morphine, but it exerts antineoplastic effects at concentrations that only temporarily block antinociception by morphine.

Conclusions

To meet criteria for pharmacological action, a given agent must elicit a characteristic response from sperm cells with appropriate dose- and time-dependent parameters. These may differ from responses observed in neurobiological systems: the dose required, the magnitude of altered action, the time course for the optimum expression should provide insights that could suggest the locus and number, as well as the accessibility, of specific receptors. Similar peculiarities have been documented in the behavior of sperm cells challenged by other well-defined agonists and antagonists.

The central action of narcotics and endogenous opioids has been attributed to interference with neurotransmission since both morphine and β-endorphin inhibit the release of acetylcholine from brain synaptosomes by preventing Ca^{2+} uptake (Sanfacon *et al.,* 1977; Guerrero-Munoz *et al.,* 1979) and naloxone blocks these effects. This test system would serve as a relevant model for interprobation of sperm cell behavior assuming that the spermatozoa possessed opioid receptors with which appropriate ligands would interact to affect the calcium-dependent acetylcholine release mechanism. We have shown that: (1) the endogenous opioid metenkephalin in ASW alters the sperm cell swimming rate biphasically; (2) (−)-naloxone, the specific opiate antagonist, in a subthreshold dose reverses metenkephalin stimulation even though by itself, (−)-naloxone also exerts a dose-dependent biphasic response; and (3) (+)-naloxone is without effect in concentrations below 1 millimolar, both by itself and when combined with metenkephalin. Thus, it appears that *Arbacia* sperm cells may indeed be equipped with opioid receptors.

Alternatively, according to recently published reports by Haynes and Smith (1982) and Haynes *et al.* (1984), the opioid peptide β-endorphin and to a somewhat lesser extent, methionine enkephalin, inhibit motor endplate-specific acetylcholinesterase in skeletal muscle of embryonic and newborn rats.

In previous studies, it has been established that the sperm cells of *Arbacia punctulata* and other species possess a functional nicotonic cholinergic system which participates in regulation of motile performance. Calmodulin-antagonists, calcium-chelators, and calcium channel blockers also have been shown to alter the rate of motile progression (Cariello and Nelson, 1985). Moreover, eserine, nicotine, and decamethonium affect the uptake and intracellular distribution of calcium in sperm cells (Nelson *et al.,* 1982). In summary, the evidence is consistent with the presence in sperm cells of opioid receptors, although somewhat atypical, and also an interdependent acetylcholine-modulated calcium-uptake system. The nature and extent of interaction of these two systems in spermatozoa remain to be elucidated.

ACKNOWLEDGMENTS

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METABOLIC ADAPTATION OF THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS*, DURING EXERCISE AND ENVIRONMENTAL HYPOXIA AND SUBSEQUENT RECOVERY*

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ABSTRACT

Metabolic responses to exercise, to exposure to environmental anaerobiosis, and to subsequent recovery were investigated in muscle, hepatopancreas, and hemolymph of the horseshoe crab, *Limulus polyphemus*. Exercise caused a considerable decline in arginine phosphate and the formation of D-lactate in muscle tissue, whereas the adenylate energy charge was maintained. Some of the D-lactate appears to have been transported from muscle tissue into the hemolymph. This occurred, apparently, during exercise as well as during recovery. Hemolymph postbranchial P_{O_2}, which decreased during exercise, and tissue phosphagen stores were rapidly restored to aerobic control values upon recovery, while D-lactate oxidation was protracted, especially in muscle.

Environmental anaerobiosis for 48 h was fueled by the breakdown of arginine phosphate (considerable only in muscle tissue) and glycogen, resulting in the accumulation of arginine, D-lactate, and alanine in both muscle and hepatopancreas. Alanine production may occur *via* glutamate-pyruvate transaminase and glutamate dehydrogenase, which take over the role of D-lactate dehydrogenase to maintain redox balance during the later phases of anaerobiosis. Recovery from anaerobiosis was characterized by a rapid replenishment of the phosphagen, a rapid drop in alanine concentration, and a protracted time-course for the decline in D-lactate levels, which was somewhat faster in the hepatopancreas than in muscle tissue.

INTRODUCTION

Hypoxia and even anoxia are encountered by many marine species. The horseshoe crab *Limulus polyphemus*, has successfully occupied microhabitats such as the estuarine intertidal zone and shallow tidal marshes, where low oxygen partial pressures may occur daily as well as a seasonally. This species is capable of extensive locomotion with its walking legs; smaller animals often swim by rhythmically moving their legs and gill leaflets (see Shuster, 1982). During excessive locomotory activity, oxygen consumption by these muscles probably exceeds the rate at which oxygen can be delivered by the circulatory system. Thus, under these conditions energy provisions are met by anaerobic metabolism (see Gäde, 1983).

Metabolic adaptations to both environmental and functional hypoxia have been studied to some extent in invertebrates, mostly molluscs, annelids, and crustaceans (for reviews see Schöttler, 1980; Gäde, 1983). During functional hypoxia, energy is typically provided by the breakdown of phosphagens (mostly arginine phosphate) and

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by anaerobic glycolysis, resulting in the production of octopine (or other opine compounds) or lactate (for reviews see Gäde, 1980; Gäde and Grieshaber, 1986). During environmental hypoxia, the phosphagen arginine phosphate again is important for ATP production. Additionally, Crustacea ferment glycogen and accumulate the classical end product L-lactate. However, in most annelids and molluscs, there is a simultaneous fermentation of glycogen and aspartate resulting in the accumulation of succinate and alanine during the first hours of hypoxia (de Zwaan and Dando, 1984). Later, when the aspartate pool is depleted, succinate production is derived exclusively from glycogen. This may be achieved by regulation at the phosphoenolpyruvate branchpoint (de Zwaan, 1977). The bulk of carbon flow is through phosphoenolpyruvate carboxykinase (PEPCK) rather than pyruvate kinase (PK). During prolonged periods of hypoxia, succinate is further metabolized to propionate.

Only a few investigations have examined the metabolic events immediately following environmental or functional anaerobiosis (see Ellington, 1983). These events include the recharging of the high energy phosphates, ATP and arginine phosphate, oxidation of end products, and resynthesis of anaerobic substrates.

Surprisingly, only scarce and scattered data on the above processes are available for the horseshoe crab. Arginine kinase is present in muscle (Blethen, 1972), indicating that arginine phosphate might contribute to energy production. The horseshoe crab possesses a D-lactate dehydrogenase (Long and Kaplan, 1968) which is present in a system of kinetically distinct, tissue-specific isoenzymes (Carlsson and Gäde, 1985). PK and PEPCK have been investigated in the tissues of *L. polyphemus* (Falkowski, 1974; Zammit and Newsholme 1978; Zammit *et al.,* 1978). The kinetic data suggest that the bulk of carbon flow is through PK rather than PEPCK (Zammit and Newsholme, 1978). Fields (1982) found that the bulk of label from anaerobic ^{14}C-glucose degradation by *in vitro* heart preparations was accumulated in lactate. Only trace amounts of radioactivity appeared in succinate.

Therefore in the present study we wanted to evaluate the overall capacity for anaerobic metabolism in muscle and hepatopancreas of *Limulus polyphemus* by estimating the relative maximal activities of enzymes of the intermediary metabolism. We were also interested in learning which metabolic events take place during exercise in muscle and hemolymph, and which take place during various periods of environmental hypoxia in muscle, hepatopancreas, and hemolymph. Finally, following the exercise stress and environmental hypoxia, we analyzed the metabolic changes in the three tissue compartments after recovery under aerobic conditions.

MATERIALS AND METHODS

Animals and tissues

Specimens of the adult horseshoe crab, *Limulus polyphemus,* (20–25 cm prosoma width) were obtained live from the Marine Biological Laboratory (Woods Hole, MA), shipped by air freight to Bonn (FRG), and kept at 15°C ± 2°C, in artificial seawater (33‰ salinity), in aquaria of 300 l. Until four days before experimentation, the animals were fed frozen fish or mussel meat once a week.

Muscle tissue from two different sites were used in this study. First, for the exercise experiments, we analyzed the muscle that moves the gill leaflets. Second, due to its small mass and its possible contribution to contractions even under hypoxic conditions, the telson levator muscle was chosen for the experiments dealing with environmental anoxia and recovery. Furthermore, the activities of key enzymes were also measured in the levator muscle, but preliminary experiments indicated that they were very similar in the gill-muscle.

In addition, a portion of the hepatopancreas from the dorsal anterior part of the animal was analyzed for enzymatic activities and changes of metabolites during environmental anoxia and recovery.

Materials

Biochemicals were purchased from Sigma Chemical Company (Taufkirchen, FRG) and Boehringer GmbH (Mannheim, FRG). All other chemicals were of reagent grade quality and came from Merck (Darmstadt, FRG). D-lactate dehydrogenase from muscle tissue of *Limulus polyphemus* was purified as described elsewhere (Carlsson and Gäde, 1985) and was used to assay D-lactate concentrations in tissues and hemolymph.

Experimental procedure

Metabolic responses to work and recovery. Animals were removed from the aquaria for different experiments, at zero time, and subjected to various periods of exercise. Horseshoe crabs held vertically in water by their telsons attempt to right themselves by making swimming movements using their legs and gills; under the conditions chosen, about 45 to 55 gill movements were made per min. After a given period of exercise (24, 47, 96, 132, 161, and 240 s), animals were removed from the tank, and the muscles that move the gill leaflets were excised and frozen in liquid nitrogen. This procedure took about 20 s. After 4 min of exercise, some animals were left to recover in well-aerated seawater for various intervals, and treated as above. Also, a zero time group was selected that had done no work at all.

Other animals were exercised for 2, 4, and 10 min, and the D-lactate concentration in the hemolymph (postbranchial, taken through the arthrodial membrane between the prosoma and the opisthosoma) was measured before and after exercise as well as at various time intervals during recovery. In some of these animals, the P_{O_2} of the postbranchial hemolymph was determined with an oxygen electrode (E 5046, Radiometer; Copenhagen, Denmark) housed in a thermostated (12 ± 2°C) cell (D 616, Radiometer). The electrode was calibrated with air-saturated seawater and with water completely depleted of oxygen by the addition of Na_2SO_3 to give a 7% solution.

All tissues were stored at −25°C.

Metabolic responses to environmental hypoxia, exposure to air, and recovery. Horseshoe crabs were incubated in Plexiglas respiration chambers filled with about 15 l seawater at 12°C which had been gassed with pure nitrogen until P_{O_2} (monitored with an oxygen electrode) reached almost zero mm Hg. After the animals were inserted, the chamber was flushed with nitrogen for another 15 to 30 min before it was closed. The chamber was placed in a constant temperature room (12 ± 1°C) for the remainder of the experiment. At the end of different incubation periods, animals were removed, a postbranchial hemolymph sample was taken, and portions of the telson levator muscle and hepatopancreas were dissected, blotted on filter paper, and immediately frozen in liquid nitrogen. An aliquot of the hemolymph sample was immediately used for P_{O_2} determination (see above), and the rest was blown into perchloric acid (see below).

Another group of horseshoe crabs was incubated in the Plexiglas respiration chamber without any seawater at 12°C (air exposure). A third group of animals was exposed to air with their gill leaflets mechanically prevented from ventilating. This was achieved by binding the gill leaflets firmly onto the body with the help of metal strips fastened with rubber bands.

Biochemical analyses of tissue and hemolymph samples

Tissue samples were fragmented with a mortar and pestle chilled in liquid nitrogen. For each analysis, approximately 200 mg of tissue of an individual muscle was weighed and homogenized in 10 volumes (w:v) 6% perchloric acid (0–4°C). The homogenates were centrifuged at 19,000 × *g* for 20 min and the supernatant fluids neutralized with 5 *M* $KHCO_3$. The neutralized extract was centrifuged as above and the supernatant immediately used for the determination of the adenylates, arginine, and arginine-phosphate. Other metabolites were determined after storage of the extracts at −25°C.

ATP was determined according to Lamprecht and Trautschold (1974), ADP and AMP after Jaworeck *et al.* (1974), arginine by the method of Gäde and Grieshaber (1975), arginine phosphate according to Grieshaber and Gäde (1976), alanine after D. H. Williamson (1974), succinate after J. R. Williamson (1974), and D-lactate according to Gawehn and Bergmeyer (1974) using the D-lactate dehydrogenase from *Limulus polyphemus.* Using conspecific D-LDH is advantageous because of its much higher affinity for D-lactate than the commercially available enzyme preparation from *Lactobacillus* (Carlsson and Gäde, 1985). No interference with heavy metal ions was observed in our lactate assay as outlined previously (Gäde, 1984).

Hemolymph samples (100–300 mg) were blown into pre-weighed vials containing 0.5 ml 6% perchloric acid; the vials were weighed again, centrifuged as above, and the supernatants were neutralized with 5 *M* K_2CO_3. After centrifugation, the supernatant was used for the D-lactate determination.

All metabolite data were analyzed for significant changes by Student's *t*-test using confidence limits of $P \leqq 0.05$.

Profile of muscle and hepatopancreas enzyme activities

Activities of key enzymes of the intermediary metabolism were estimated in crude, desalted, cell-free extracts of the telson levator muscle and hepatopancreas of *L. polyphemus.* Approximately 1 g of tissue was cut into small pieces, resuspended in a 5-fold volume (w:v) of extraction buffer [100 m*M* triethanolamine-HCl (TRA) buffer containing 1 m*M* 2-mercaptoethanol at pH 7.6], homogenized by sonification (Branson sonifier), and centrifuged at 20,000 × *g* for 20 min. The supernatant was passed through a Sephadex G-25 column (Deutsche Pharmacia GmbH, Freiburg, FRG) to remove low molecular weight compounds.

The enzymes were assayed by standard procedures reported in the literature. The assay conditions have been outlined in detail previously (Meinardus-Hager and Gäde, 1986). Exceptions are the reaction mixtures (final concentration in a 1 ml cuvette) of the following enzymes:

Alcohol dehydrogenase (EC 1.1.1.1): 85.5 m*M* sodium pyrophosphate buffer pH 9, 19 m*M* glycine, 6.2 m*M* semicarbazid-hydrochloride, 0.6 *M* ethanol, 1.8 mM NAD^+, 1 m*M* glutathion.

D-lactate dehydrogenase (LDH, EC 1.1.1.28): 100 m*M* potassium phosphate buffer pH 7.0, 0.15 m*M* NADH, start with 2.5 m*M* pyruvate (1 m*M* pyruvate in case of extracts from hepatopancreas).

Glutamine synthetase (EC 6.3.1.2): 200 m*M* Tris/HCl pH 7.7, 100 m*M* hydroxylammoniumchloride, 10 m*M* Na_2HAsO_4, 0.5 m*M* $MnSO_4$, 1.5 m*M* ADP, 100 m*M* glutamine. After 30 min the reaction was stopped by addition of an acidic solution of $FeCl_3 \times 6\ H_2O$ and the extinction measured at 546 nm.

All assays were conducted in a filter photometer (Vitatron DCP 4) or a LKB Ultrospec recording spectrophotometer at 25°C. Assays were initiated by the addition of substrate.

RESULTS

Profile of the activities of key enzymes in the intermediary metabolism

The activities of some enzymes involved in the energy metabolism in muscle and hepatopancreas tissue of *L. polyphemus* are listed in Table I. Glycogen phosphorylase in the muscle was only 6% in the a-form, while in the hepatopancreas the a-form of the enzyme represented 46% of the total enzyme activity. In the muscle tissue, the activity of the hexokinase was about 7-fold higher than that of the activated phosphorylase, whereas no significant difference between these two enzymes was observed in the hepatopancreas. Glyceraldehyde-3-phosphate dehydrogenase of the muscle had the highest activity of the enzymes of the Embden-Meyerhof pathway followed by pyruvate kinase and phosphofructokinase. This was true also for hepatopancreas, but

TABLE I

Activity of various enzymes in telson levator muscle and hepatopancreas from Limulus polyphemus

		Enzyme activity (µmoles substrate conversion per min and g wet weight)	
Enzyme	(n)	Telson levator muscle	Hepatopancreas
Enzymes involved in glycolysis:			
Phosphorylase a-form	(2)	0.022 ± 0.005	0.13 ± 0.03
a + b-form		0.34 ± 0.10	0.28 ± 0.06
Hexokinase	(4)	2.32 ± 0.45	0.39 ± 0.16
Phosphofructokinase	(3)	29.9 ± 6.2	1.22 ± 0.12
Glyceraldehydephosphate dehydrogenase	(3)	136 ± 12	10.5 ± 2.9
Pyruvate kinase	(3)	73.3 ± 13	5.6 ± 1.4
Lactate dehydrogenase	(4)	102 ± 1	29.3 ± 5.1
Octopine dehydrogenase	(3)	n.d.*	n.d.
Strombine dehydrogenase	(3)	n.d.	n.d.
Alanopine dehydrogenase	(3)	n.d.	n.d.
Alcohol dehydrogenase	(3)	n.d.	n.d.
Enzyme involved in phosphagen utilization:			
Arginine kinase	(3)	616 ± 17	34.0 ± 9.6
Citric acid cycle enzymes:			
Citrate synthase	(2)	2.05 ± 0.28	1.49 ± 0.08
Malate dehydrogenase	(4)	156 ± 15	91 ± 7
Enzymes involved in amino acid metabolism:			
Glutamate dehydrogenase			
NADH-dependent	(4)	1.74 ± 0.24	0.69 ± 0.12
NADPH-dependent	(4)	n.d.	n.d.
Glutamine synthetase	(2)	0.027 ± 0.008	0.081 ± 0.013
in the presence of 10 m*M* alanine	(2)	0.018 ± 0.005	0.005 ± 0.016
Glutamate-oxaloacetate transaminase	(3)	28.9 ± 3.7	19.1 ± 7.7
Glutamate-pyruvate transaminase	(3)	13.5 ± 1.0	16.7 ± 2.4
Enzymes involved in gluconeogenesis:			
Fructose diphosphatase	(3)	0.74 ± 0.19	2.04 ± 0.18
Phosphoenolpyruvate carboxykinase	(3)	1.5 ± 0.3	2.1 ± 0.5

* n.d. = not detectable.

the maximum activities were 10- to 20-fold lower than in muscle tissue. Of the four terminal pyruvate reductases measured in both tissues, only D-lactate dehydrogenase was present. There was no activity at all of octopine-, alanopine-, and strombine dehydrogenase. The activity of D-lactate dehydrogenase in muscle tissue was almost as high as the activity of glyceraldehyde-3-phosphate dehydrogenase and, in hepatopancreas, 3-fold of the latter activity.

Fermentation of glycogen to ethanol occurs in anoxic muscle of fishes (Shoubridge and Hochachka, 1980) and in tissues of larvae of the insect *Chironomus thumii thumii* (Wilps and Zebe, 1976). Due to the absence of activity of alcohol dehydrogenase in tissues of *L. polyphemus,* this type of fermentation cannot take place.

Arginine kinase activity was very high in muscle tissue, but the hepatopancreas displayed only a fraction of this activity. Malate dehydrogenase activity was very high in both tissues, and there was a significant activity of citrate synthase in both tissues.

Activity of phosphoenolpyruvate carboxykinase, which catalyses the fixation of CO_2, could readily be measured in both tissues, but activity was slightly higher in the hepatopancreas. This was also true for the enzyme fructose-biphosphatase, an enzyme thought to operate during gluconeogenesis.

Enzymes involved in amino acid metabolism were also assayed in both tissues. The transaminases showed the highest activities. Glutamate dehydrogenase in both tissues was only active with NADH, and not NADPH, as the co-substrate. Very low activities of glutamine synthetase were found in both tissues (but they were higher in hepatopancreas); this enzyme was inhibited by the addition of 10 m*M* alanine to the reaction mixture.

Metabolic responses to exercise and recovery

When specimens of *L. polyphemus* were subjected to exercise, there was a linear relationship between the numbers of gill movements and time (Fig. 1, inset). The level of D-lactate in the gill-muscle tissue increased linearly until up to 3 min and then plateaued (Fig. 1). After 20 and 30 min of recovery from 4 min of exercise, the D-lactate levels were significantly lower than the exercise value, but the control levels were not attained. There was no change in the alanine concentration during work (Fig. 1). Exercise also had no significant effect on the adenylates in the muscle tissue and, consequently, the adenylate energy charge did not change (Table II). In contrast, arginine phosphate levels fell significantly during exercise (Fig. 2). The most pronounced change occurred during the first 47 s of exercise. The increase in free arginine levels was a mirror image of the pattern of change in arginine phosphate levels (Fig. 2). During recovery, arginine phosphate levels rose rapidly and reached initial levels after about 20 to 30 min. At the same time there was a decrease in the arginine concentration. There were no changes in aspartate and succinate levels in any of these experimental treatments (results not shown).

In the hemolymph, D-lactate concentrations did not change during 2 min of exercise, whereas concentrations rose significantly during recovery (Fig. 3): the highest D-lactate concentrations were observed after 5 min of recovery. After 90 min of recovery, hemolymph D-lactate concentrations were not different from controls. After 4 and 10 min of exercise, the D-lactate concentrations in the hemolymph were significantly increased above control concentrations, however, the concentrations at the two times were not significantly different from each other. During recovery from 4 and 10 min of work, there was an apparent large increase of the D-lactate concentration in the hemolymph; however, due to the inherent variability of the measurement, this increase was not significantly different from the values obtained just after work.

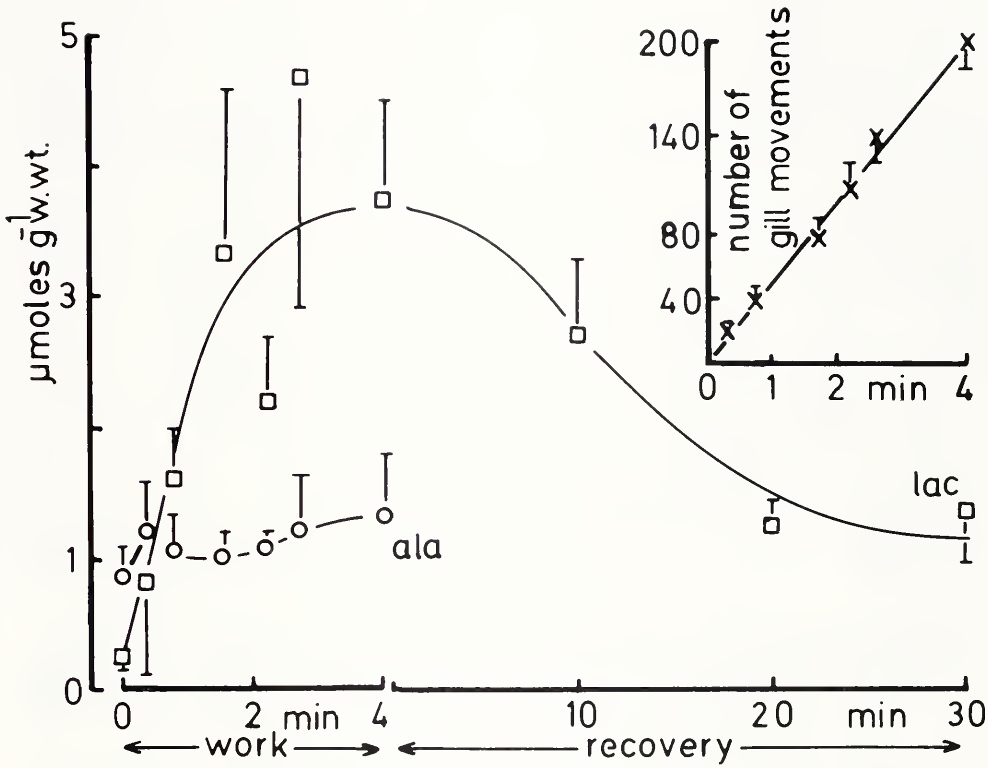

FIGURE 1. Alterations in the levels of D-lactate (□ — □) and alanine (O — O) in muscle that moves the gill leaflets of *Limulus polyphemus* during exercise and recovery. Each value is a mean ± SD (n = 4). The inset shows the relationship of the numbers of gill movements *versus* time.

P_{O_2} levels of the hemolymph decreased after 4 min of exercise, but were already back to control levels after 10 min of recovery (Fig. 4).

Metabolic responses to environmental hypoxia, exposure to air, and recovery

In a first series of experiments we analyzed the oxygen partial pressure of post-branchial hemolymph and the blood D-lactate concentration after subjecting specimens

TABLE II

Alterations in the levels of adenylates (µmoles per g wet weight) and the calculated energy charge in muscle that moves gill leaflets of Limulus polyphemus *during different durations of exercise*

Time (s)	(n)	ATP	ADP	AMP	Sum	Energy charge
0	3	2.73 ± 0.60	0.42 ± 0.12	0.03 ± 0.01	3.18	0.92
23.6 ± 4.5	3	2.47 ± 0.49	0.42 ± 0.16	0.06 ± 0.03	2.95	0.91
46.7 ± 7.9	3	3.24 ± 0.72	0.51 ± 0.15	0.04 ± 0.01	3.95	0.92
95.6 ± 9.0	3	2.60 ± 0.53	0.44 ± 0.10	0.03 ± 0.02	3.07	0.92
161.0 ± 8.2	3	2.56 ± 0.24	0.48 ± 0.14	0.04 ± 0.01	3.08	0.91
240.5 ± 26.7	3	2.44 ± 0.44	0.46 ± 0.19	0.05 ± 0.01	2.95	0.91

Each value is a mean ± SD.

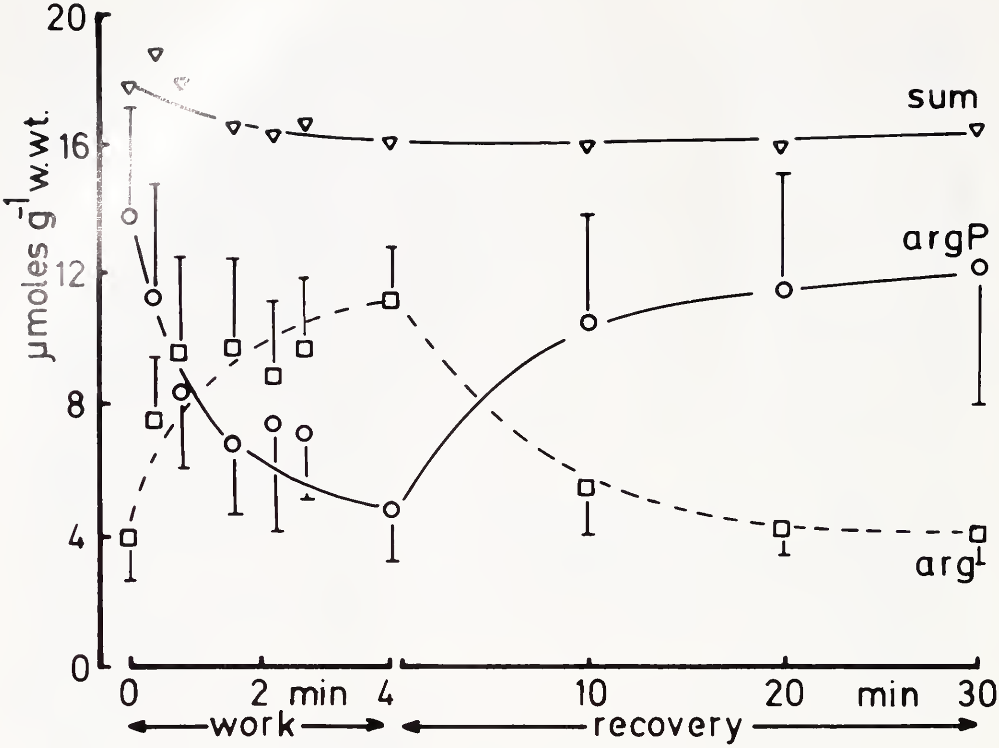

FIGURE 2. Changes in the levels of arginine phosphate (○ — ○) and arginine (□ — □) and the sum of these metabolites (▽ — ▽) in muscle that move the gill leaflets of *Limulus polyphemus* during exercise and recovery. Each value is a mean ± SD (n = 5).

of *L. polyphemus* to extremely hypoxic seawater for various periods of time (Fig. 5A); and during air exposure for 72 h (Fig. 5B); and air exposure for 48 h when the gills were mechanically prevented from ventilating (Fig. 5C).

Incubation in oxygen-free seawater and exposure to air with unventilated gills produced the same general results (Fig. 5A *versus* 5C): postbranchial blood P_{O_2} decreased to low levels after only a short incubation period, and blood D-lactate levels rose to about 3 µmoles/g wet weight of hemolymph during the first two hours and stayed almost contant thereafter. During air exposure with intact gills (Fig. 5B), a decrease in postbranchial P_{O_2} was observed after 2 h, but thereafter it rose again to about 30 mm Hg. D-lactate levels increased somewhat during the first 2 h but were back to control levels after 48 h of air exposure. During recovery from a 48 h period of incubation in oxygen-free seawater (Fig. 5A), P_{O_2} levels reached control values within 30 min of recovery, and after 1 and 2 h of recovery were slightly higher than P_{O_2} values observed under control conditions. The hemolymph D-lactate levels showed a trend towards an increase during the first hour of recovery (Fig. 5A), but due to the high variation this was not significant. After 8 h of recovery, the D-lactate levels were still higher than control values.

Incubation in oxygen-free seawater had no significant effect on the adenylate concentrations and the energy change in muscle, but ATP levels fell and ADP levels rose significantly in hepatopancreas after 8 h of incubation (Table III). After 48 h of hypoxia

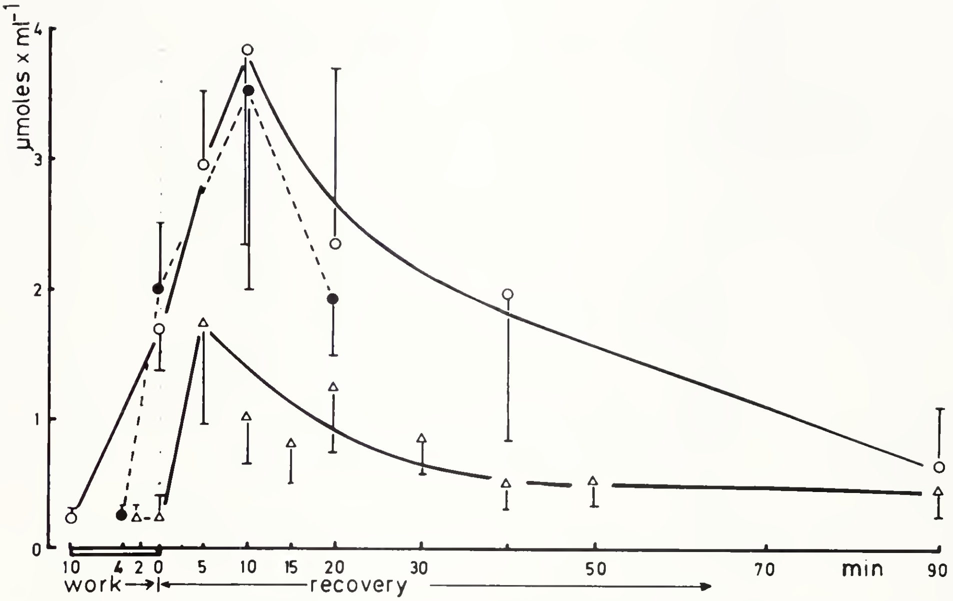

FIGURE 3. Time course of the levels of D-lactate in the hemolymph of *Limulus polyphemus* during different durations of exercise (2 min: △ — △; 4 min: ● — ●; 10 min: ○ — ○) and subsequent recovery. Each value is a mean ± SD (n = 4).

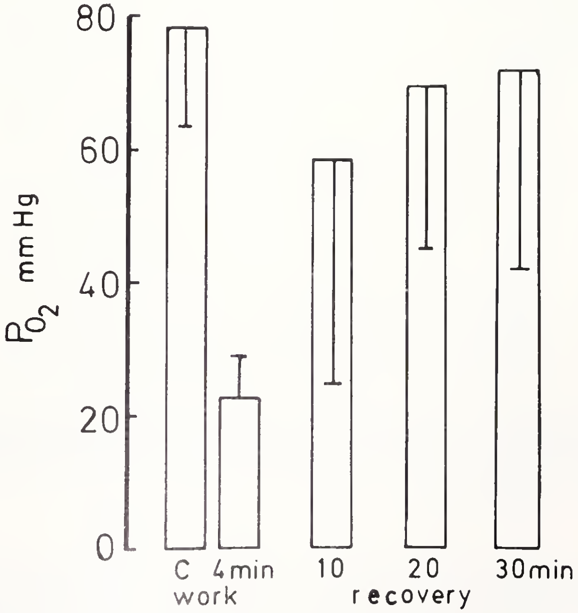

FIGURE 4. Changes in postbranchial P_{O_2} in the hemolymph of *Limulus polyphemus* during work and recovery. Each value is a mean ± SD (n = 4).

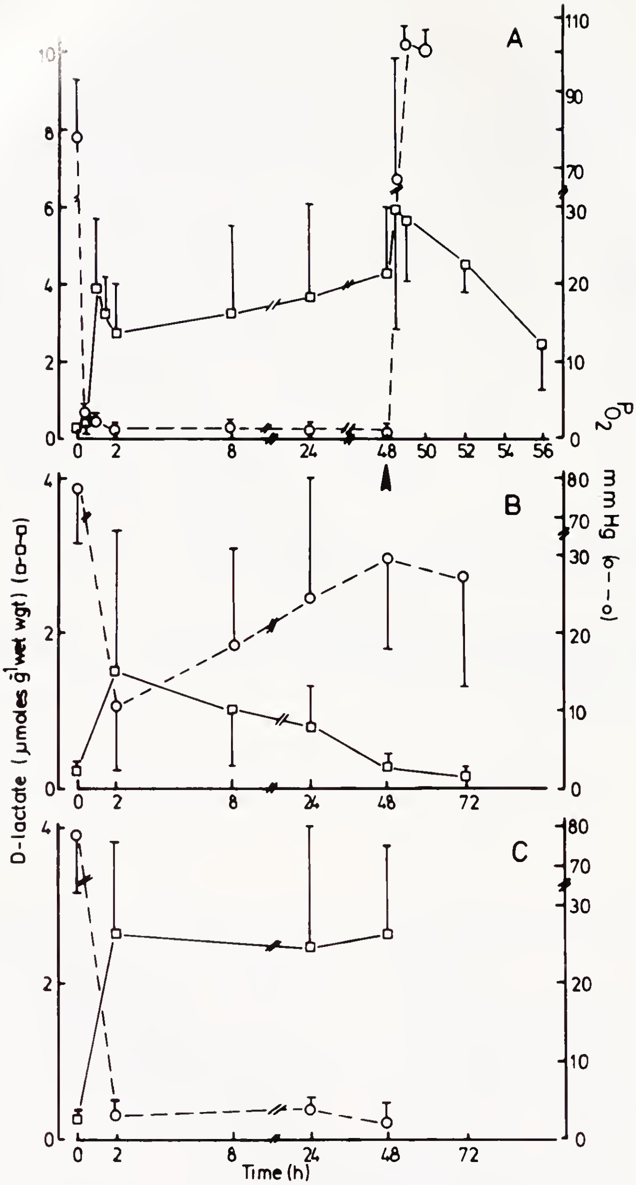

FIGURE 5. Effect of incubation in oxygen-free seawater (A), air exposure (B) and air exposure with mechanically closed gills (C) as well as recovery (A only) therefrom of the level of hemolymph D-lactate (□ — □) and postbranchial P_{O_2} (O — O) in *Limulus polyphemus*. Onset of recovery is indicated by an arrow. Each value is a mean ± SD (n = 4).

only one third of the control ATP levels remained, while ADP levels were 3-fold above the control levels. The adenylate energy charge fell from 0.86 (control) to 0.61 after 48 h of hypoxia. In the muscle, arginine phosphate levels fell significantly during hypoxia with the greatest changes occurring in the first 10 h (Fig. 6). Although there

TABLE III

Effect of incubating Limulus polyphemus *in oxygen-free seawater on the levels of adenylates (μmoles per g wet weight) and the calculated energy charge in the telson levator muscle and hepatopancreas*

	Telson levator muscle					Hepatopancreas				
Time (h) of anoxia	ATP	ADP	AMP	Sum	Energy charge	ATP	ADP	AMP	Sum	Energy charge
0.0	2.82 ± 0.65	0.48 ± 0.14	0.05 ± 0.21	3.35	0.91	0.47 ± 0.12	0.10 ± 0.02	0.03 ± 0.01	0.60	0.86
0.5	2.69 ± 0.26	0.59 ± 0.21	0.05 ± 0.02	3.33	0.90	0.33 ± 0.05	0.09 ± 0.05	0.01 ± 0.01	0.44	0.86
2.0	2.43 ± 0.20	0.53 ± 0.16	0.06 ± 0.03	3.02	0.89	0.37 ± 0.11	0.13 ± 0.02	0.03 ± 0.01	0.53	0.82
4.0	2.98 ± 0.43	0.60 ± 0.07	0.06 ± 0.01	3.64	0.90	0.35 ± 0.14	0.11 ± 0.03	0.04 ± 0.01	0.50	0.81
8.0	2.80 ± 0.13	0.67 ± 0.06*	0.04 ± 0.02	3.51	0.89	0.27 ± 0.08*	0.24 ± 0.04*	0.02 ± 0.01	0.42	0.74
24.0	2.73 ± 0.49	0.79 ± 0.04*	0.05 ± 0.02	3.57	0.88	0.26 ± 0.04*	0.24 ± 0.09*	0.03 ± 0.01	0.53	0.72
48.0	2.81 ± 0.18	0.67 ± 0.07*	0.07 ± 0.02	3.55	0.89	0.14 ± 0.02*	0.28 ± 0.12*	0.04 ± 0.02	0.46	0.61

* Significantly different to control value.
Each value is a mean ± SD (n = 4).

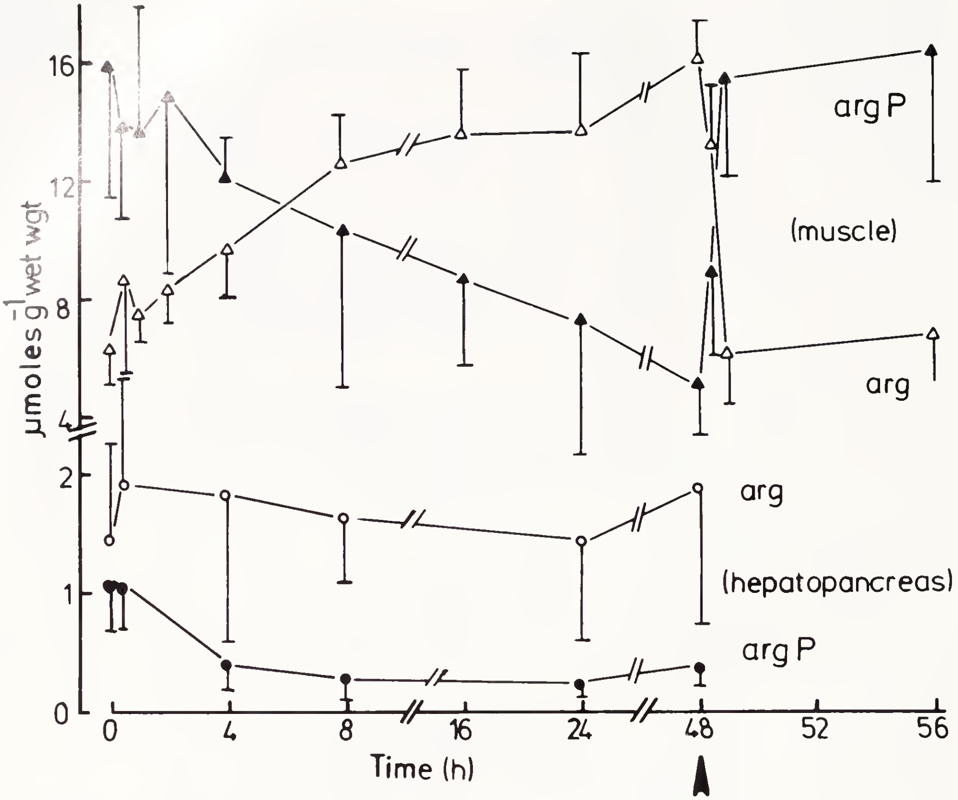

FIGURE 6. Effect of incubating *Limulus polyphemus* in oxygen-free seawater for various times on the levels of arginine phosphate and arginine in the telson levator muscle and hepatopancreas. The time course of changes occurring during recovery in muscle is also shown; onset of recovery is indicated by the arrow. Each value is a mean ± SD (n = 5).

was some breakdown of arginine phosphate in the hepatopancreas during hypoxia, quantitatively this did not contribute much to overall energy production due to the low initial levels of this compound (Fig. 6). During recovery after hypoxia, arginine phosphate levels in muscle returned to initial values within 1 h.

There were significant changes in the levels of D-lactate and alanine during hypoxia in both muscle and hepatopancreas tissue. After an initial increase in the first hour, D-lactate levels in muscle tissue as well as in hepatopancreas seemed to increase slightly with time, but due to the high variability these changes were not significant (Fig. 7). Until 2 h of recovery had passed, D-lactate levels in muscle remained high, and they were still above control levels after 8 h of recovery. In the hepatopancreas, the decrease in D-lactate levels was somewhat faster, yet control values were not reached after 8 h of recovery.

L-alanine levels increased with time of hypoxia in both tissues (Fig. 8). However, in muscle tissue most of the alanine was produced until 24 h of hypoxia, whereas there was a further increase in alanine levels between 24 and 48 h of hypoxia in the hepatopancreas. Succinate production was minimal during the first 10 h of hypoxia in the muscle tissue and was absent in hepatopancreas (Fig. 8). Aspartate levels of

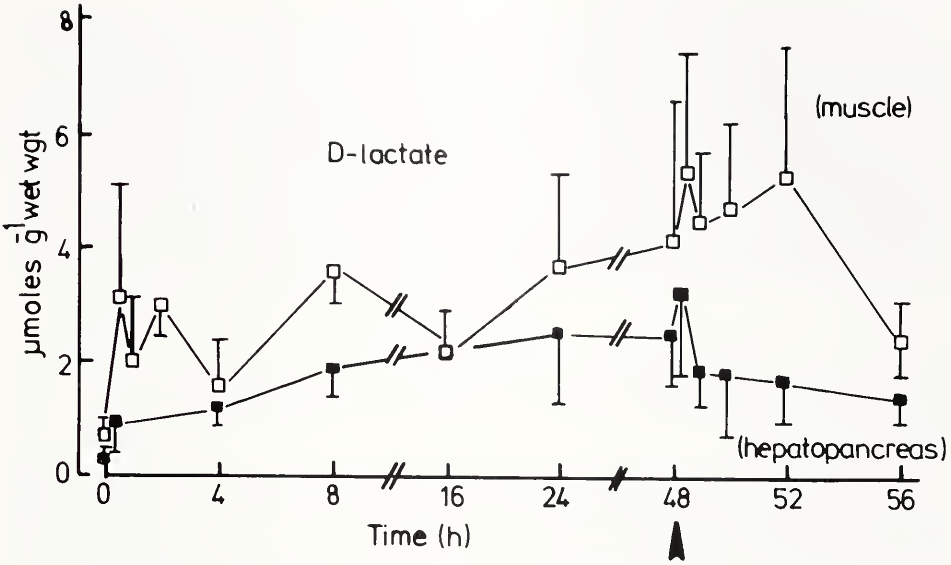

FIGURE 7. Effect of incubating *Limulus polyphemus* in oxygen-free seawater for various times on the level of D-lactate in the telson levator muscle and hepatopancreas. The time course of changes occurring during recovery is also shown; onset of recovery is indicated by the arrow. Each value is a mean ± SD (n = 4).

muscle and hepatopancreas were 0.29 ± 0.11 and 0.24 ± 0.06 µmoles/g wet weight, respectively, in control animals and 0.13 ± 0.06 and 0.07 ± 0.04 µmoles/g wet weight, respectively, after 48 h of hypoxia (for all data: n = 4).

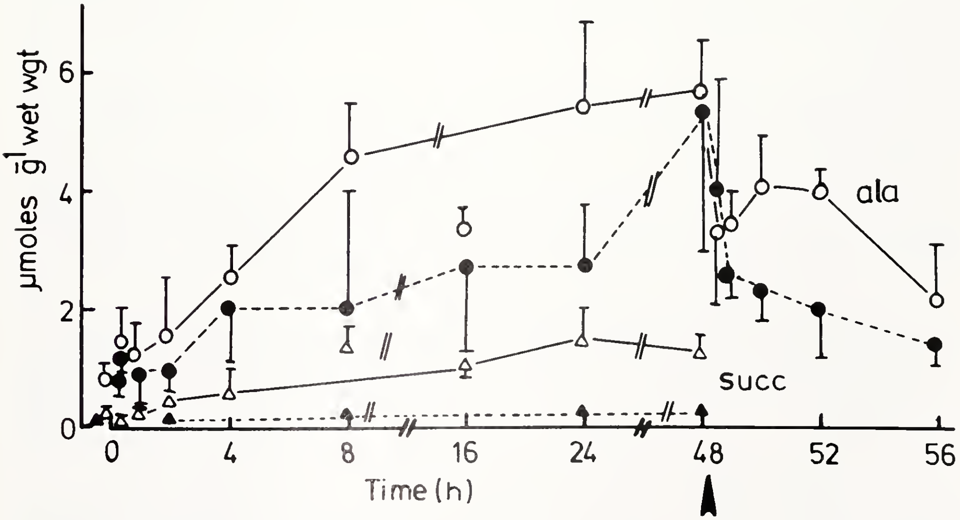

FIGURE 8. Effect of incubating *Limulus polyphemus* in oxygen-free seawater for various times on the level of alanine (○, ●) and succinate (△, ▲) in the telson levator muscle (open symbols) and hepatopancreas (closed symbols). The time course of changes in alanine levels occurring during recovery is also shown; onset of recovery is indicated by the arrow. Each value is a mean ± SD (n = 4).

During recovery from hypoxia, alanine levels fell in both muscle and hepatopancreas. However, the time course of alanine removal was much more rapid in the hepatopancreas (Fig. 8). The alanine concentration in the postbranchial blood was below 0.05 μmoles/g wet weight throughout the whole experiment (hypoxia and recovery). Muscle, hepatopancreas, and hemolymph glutamate levels (1.59 ± 0.58, 3.30 ± 1.22, 0.04 ± 0.02 μmoles/g wet weight, respectively) did not change significantly during the experiment, whereas muscle and hepatopancreas glutamine levels decreased significantly from 1.21 ± 0.42 and 2.84 ± 1.13 μmoles/g wet weight, respectively, to 0.33 ± 0.21 and 0.55 ± 0.03 μmoles/g wet weight, respectively. Control levels of glutamine in both tissues were not attained again until after 8 h of recovery.

Discussion

Scope of aerobic and anaerobic energy metabolism as estimated by maximal enzyme activities

The capacity for aerobic and anaerobic energy metabolism of the telson levator muscle and hepatopancreas of *L. polyphemus* was first estimated by the determination of relative enzyme activities of the intermediary metabolism. Such a comparison may be instructive in trying to understand potential differences in the metabolism of *L. polyphemus* muscle and hepatopancreas tissue. In muscle tissue, arginine phosphate and glucose primarily and glycogen to a lesser extent, may be considered fuels for energy production. This is indicated by the following data. The enzyme arginine kinase is present in very high catalytic activities. In fact, the activity is substantially higher than the glycolytic enzyme activities. Accordingly, the phosphagen can be broken down quite rapidly, even when the energy demand is much enhanced as it is during muscular activity. Although glycogen phosphorylase (and therefore glycogen breakdown) can be activated about 16-fold by AMP, hexokinase activity still is about 7-fold higher. This may indicate that during periods of enhanced energy demand, exogenous glucose from the hemolymph may be a more important carbon source than glycogen.

In the hepatopancreas the enzyme data suggest that glycogenolysis and glucose metabolism may contribute equally to energy production, whereas the breakdown of phosphagen plays only a minor role.

In both tissues, especially in muscle, the other glycolytic enzymes known to catalyse rate-limiting reactions, phosphofructokinase (PFK) and pyruvate kinase (PK) have high activities, and exceed that of phosphorylase from 4- to 31-fold. This indicates, that the initial enzymes of glycolysis (phosphorylase, hexokinase) most probably catalyze the reactions which are rate-limiting steps in this pathway.

The expected anaerobic end-product is D-lactate. Both tissues contain only this terminal dehydrogenase and the activity is quite high, even in the hepatopancreas.

A representative enzyme of the citric acid cycle, citrate synthase, is present in both tissues at activity levels which are much higher than observed in most anoxia-tolerant invertebrate tissues (see, for example, Pörtner *et al.,* 1984; Meinardus-Hager and Gäde, 1986) suggesting a high aerobic capacity.

The relatively high activities of enzymes thought to be involved in gluco- and glyconeogenesis, phosphoenolpyruvate carboxykinase, and fructose-bisphosphatase, indicate that this pathway might be working during recovery after anaerobiosis to recharge the depleted stores of glycogen and glucose as was indeed shown recently (Gäde *et al.,* 1986).

Energy metabolism during swimming and recovery

The significance of the above discussed enzymatic patterns for the anaerobic production of energy was shown in individual horseshoe crabs which were made to swim for different periods. During exercise, the aerobic capacity of the muscle tissue was not sufficient to meet the enhanced energy demand. Pronounced anaerobic synthesis of ATP was achieved by the production of D-lactate and, second, by the breakdown of the phosphagen. This rate of anaerobic ATP production appears to have been sufficient to meet the energy demands as shown by the unchanged energy charge. In crustaceans, however, the energy charge is lowered during vigorous activity (Onnen and Zebe, 1983; Gäde, 1984).

D-lactate produced in muscle tissue was only accumulated until a concentration of about 3–4 μmoles per g wet weight was reached, which occurred after only 2 min of work. During prolonged exercise muscle-produced D-lactate appears to have been released into the hemolymph. Unexpectantly, the hemolymph D-lactate concentration was not significantly different after 4 and 10 min work. This was also true during recovery after these periods of exercise, when in both cases a further increase of D-lactate was measured. The lack of difference between these exercised groups could be due to variability in the data resulting from the measurement of a transient in blood. Alternatively, the work output between 4 and 10 min may be lowered and, thus, aerobic energy production may contribute to a greater extent.

Oxidation of D-lactate during recovery is very protracted, so tissues other than muscle may be contributing. The hepatopancreas, for example, contains D-LDH isoenzymes, which are kinetically well suited for lactate oxidation (Carlsson and Gäde, 1985). In contrast, replenishment of pre-work arginine phosphate levels during recovery is rapid and probably achieved aerobically due to the effectiveness of the circulatory system.

Energy metabolism during environmental hypoxia, exposure to air and recovery

Incubation in oxygen-free seawater and air exposure with mechanically closed gills induce horseshoe crabs to rely on anaerobic metabolism for their total energy production; the hemolymph P_{O_2} declined to about zero and D-lactate levels increased. As no differences are found between these two anaerobic groups it is clear that excretion of D-lactate does not occur during incubation in oxygen-free water. Air exposure with intact gill function was tolerated for at least three days without significant D-lactate production, indicating the absence of anaerobic metabolism. The reason lies partly in the function of the gills and partly in the unique properties of the oxygen carrier, hemocyanin. Oxygenated hemocyanin-transport was calculated to be enhanced during air exposure by 24% due to the reverse Bohr-shift (Mangum *et al.,* 1975; Mangum, 1983).

In the telson levator muscle, the anaerobic energy metabolism was efficient enough to meet the energy demand during severe hypoxia for 48 h. There was no change in the levels of adenylates and, consequently, of the energy charge. ATP production was achieved by transphosphorylation of the phosphagen and breakdown of glycogen resulting in the production of D-lactate.

In contrast, due to the low initial concentrations of arginine phosphate, the energy charge decreased considerably in the hepatopancreas. Again, ATP was provided by the formation of D-lactate. However, in both tissues, the amount of D-lactate which accumulated never exceeded 4 μmoles per g wet weight, suggesting that another end product was formed by another route of anaerobic energy production.

A likely candidate was the succinate/propionate route well-known from euryoxic bivalves and annelids (for reviews see Gäde, 1983; Schöttler, 1980), but not operating to a meaningful extent in crustaceans. However, the succinate levels in muscle and hepatopancreas tissue were only elevated by 1 and 0.1 μmol/g wet weight, respectively, and propionate was not present at all. Other data support the notion that the succinate/propionate pathway is not working in *L. polyphemus* (*e.g.*, Fields, 1982; Zammit and Newsholme, 1978).

Which other route of energy production, besides arginine phosphate transphosphorylation and D-lactate formation, might occur then in *L. polyphemus?* Muscle and hepatopancreas accumulate L-alanine consistently. The bulk of this accumulation is observed after the main D-lactate formation has already occurred, and it quantitatively exceeds the D-lactate accumulation. Alanine production, *via* GOT and GPT, is known in bivalves and annelids, but is not possible in *L. polyphemus* due to the low levels of aspartate. We therefore propose a mechanism with the participation of GPT and glutamate dehydrogenase (see Fig. 9). Pyruvate derived by anaerobic breakdown of glycogen would be converted to alanine and α-ketoglutarate by the action of GPT. The keto acid, in turn, is oxidized with NH_4^+ by glutamate dehydrogenase to regenerate glutamate for the GPT reaction and to produce the oxidized coenzyme NAD^+. The

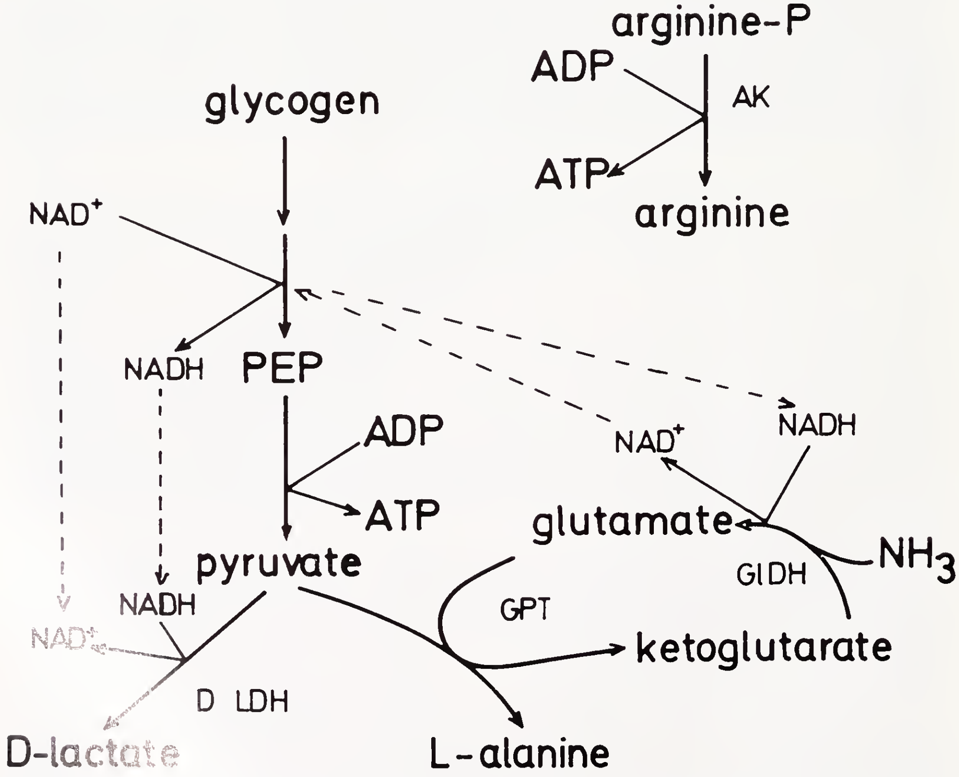

FIGURE 9. Proposed scheme of anaerobic energy metabolism in *Limulus polyphemus.* Note: no compartmentation (cytosol-mitochondria) is given.

only end product of this reaction would be alanine. Our experimental data support this hypothesis, as follows.

(1) The maximal activities of GPT and GluDH are sufficient to account for the observed rates of accumulation of alanine.

(2) Partially purified GluDH from *L. polyphemus* muscle tissue is exclusively NADH-, but not NADPH-dependent. In addition, GluDH and D-LDH from *L. polyphemus* appear to have similar catalytic affinities for NADH (Carlsson and Gäde, 1985, and unpub.).

For this reaction scheme to operate, GluDH must be localized in the cytoplasm where it is stoichiometrically coupled with the glyceraldehyde-3-phosphate dehydrogenase reaction. As yet, however, we have no data on the localization of GluDH in *L. polyphemus* tissue.

What is the advantage for the horseshoe crab to rely on alanine production via GluDH instead of a continuous formation of D-lactate? D-LDH is an equilibrium enzyme. The K_m-values for D-lactate are in the range of 10 m*M* for the muscle isoenzymes and even lower, 5 m*M*, for the hepatopancreas isoenzymes (Carlsson and Gäde, 1985). Thus, from a kinetic point of view, D-lactate cannot accumulate to high levels, since the reaction is shifted back then to D-lactate oxidation. In contrast, the K_m-value for L-lactate in the crayfish *O. limosus* is about 40 m*M* (Urban, 1969), and here we found L-lactate levels as high as 16 m*M* in muscle tissue after anaerobiosis (Gäde, 1984). In *L. polyphemus*, D-lactate production occurs mainly during the beginning of anaerobiosis; but later, when the D-lactate levels may hamper further reduction of pyruvate, NADH oxidation is achieved by the action of GluDH. The production of alanine may serve at least two purposes:

(1) The enzyme pyruvate kinase is inhibited by 85% in the presence of 5 mM alanine (Zammit and Newsholme, 1978; our unpub. results), and, therefore, glycolytic flux is reduced during prolonged anaerobiosis, as observed in bivalves and annelids (see, Gäde, 1983; Schöttler, 1980). Calculations of rates of anaerobic energy production in muscle tissue of *L. polyphemus* from our metabolic data (1.5 μmol of ATP per μmol of D-lactate and L-alanine produced) revealed a rate of 0.0465 μmol ATP/g wet weight/min during the first two hours of anaerobiosis, in contrast to 0.0036 during 16 to 48 hours of anaerobiosis.

(2) In mammals, the enzyme glutamine synthetase is inhibited by alanine (see Meister, 1974). This enzyme synthesizes glutamine from glutamate and NH_{4+} coupled with the consumption of ATP. Glutamine, in turn, is important for the synthesis of, for example, purines, pyrimidines, carbamylphosphate, and various proteins. Inhibition of this enzyme, therefore, would channel glutamate and NH_{4+} away and hamper the energy-expending synthesis of nitrogen compounds. Our preliminary data on crude extracts of horseshoe crab muscle and hepatopancreas glutamine synthetase showed inhibition by alanine and is thus in agreement with the arguments above. During recovery of *L. polyphemus* from 48 hours of anaerobiosis a rapid drop in the levels of alanine in muscle and hepatopancreas might relieve the inhibition of both glycolysis and glutamine synthetase.

In summary, the horseshoe crab is well equipped to cope with environmental anaerobiosis. Metabolic energy is derived by the breakdown of phosphagen and by the production of D-lactate and, later, alanine. In the latter phase GluDH may take over the role of D-LDH to maintain redox-balance. In contrast, aside from the transphosphorylation reaction, crustaceans use only anaerobic glycolysis leading to L-lactate accumulation as means of providing energy during low oxygen tension.

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THE MECHANICS OF FISH SKIN: LACK OF AN "EXTERNAL TENDON" ROLE IN TWO TELEOSTS

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ABSTRACT

The skin of the Norfolk spot, *Leiostomus xanthurus,* is composed largely of a crossed-helical array of collagen fibers. Over most of the body of the fish these are oriented at angles of 45–80° with the long axis of the fish. The skin of the skipjack tuna, *Katsuwonus pelamis,* also contains a crossed-helical array of collagen fibers, although fewer fiber layers are present and fiber angles are generally in the range of 55–75°. Uniaxial stress-strain tests indicate that for both species skin is most extensible in the longitudinal direction. For the Norfolk spot, skin is stiffer in the direction of the fibers than in the circumferential direction, but for the skipjack tuna, the skin is of about the same stiffness in the circumferential direction as it is in the direction of the fibers. Biaxial stressing tests demonstrate that the skins of the spot and the skipjack do not behave as simple crossed-fiber systems, and are therefore incapable of transmitting forces down the lengths of these fishes or acting as "external" tendons.

INTRODUCTION

The skin of fishes is composed largely of a system of collagen fibers that form alternating layers of right and left helices wrapped about the long axis of the animal. This arrangement of collagen fibers simultaneously provides a supporting framework to enclose the body contents and a flexible covering to allow whatever changes in shape are necessary during locomotion. This crossed-fiber arrangement has been described in elasmobranchs (Motta, 1977; Wainwright *et al.,* 1978) and teleosts (Fujii, 1968; Nadol *et al.,* 1969; Brown and Wellings, 1970; Hawkes, 1974; Willemse, 1972; Videler, 1975; and Hebrank, 1980).

Engineers use this design feature for reinforcing thin-walled pressurized cylinders by winding them with inextensible fibers. With this design a lightweight cylinder wall is created that can strongly resist internal pressures, yet is capable of extensions in any direction except those of the fibers themselves. In the directions of the circumference and long axis of the cylinder, large extensions are permitted (these are the directions that bisect the angles between a pair of crossed-helices) and because the fibers are inextensible, torsion of the cylinder is also strongly resisted. The angle that the helices make with the long axis of the cylinder is called the fiber angle, and for a range of fiber angles excluding those near 0° and 90°, the cylinder can be bent without kinking or wrinkling. The convex side of a bent constant volume cylinder is stretched in the longitudinal direction; on this side the fiber angle decreases. The reverse occurs on the opposite or concave side. In this way the cylinder (or a fish) can undergo a range

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of movements without changing its body form or volume (Clark, 1964) and without kinking or wrinkling, which is important to hydrodynamic stability.

The importance of a crossed-fibrillar array of collagen to fish locomotion was first demonstrated by Wainwright *et al.* (1978) with their study of shark skin. These authors found the skin to be capable of acting as an external tendon, transmitting force and displacement in parallel with the attached, underlying muscles. Because a force applied to the skin can generate a greater bending moment than the same force applied near the backbone, the mechanical advantage of the axial musculature can be enhanced by pulling on skin that is capable of transmitting forces down the length of the fish. A subsequent study by Hebrank (1980) concluded that the skin of the American eel (*Anguilla rostrata*) is also capable of serving as an external tendon.

Sharks and eels, however, are considered to be relatively primitive fishes, and both swim using fairly large amplitude waves of lateral undulation. In this study the structural features and mechanical properties of the integuments of two more advanced teleosts, the Norfolk spot (*Leiostomus xanthurus*) and the skipjack tuna (*Katsuwonus pelamis*), were studied in a manner similar to those used to investigate the mechanics of shark and eel skin. Neither of these fishes exhibits the degree of lateral undulation in swimming characteristic of eels and sharks, therefore the purpose of this study was to determine whether the skins of these two teleosts are capable of serving as external tendons.

MATERIALS AND METHODS

Experimental animals

Five Norfolk spots (*Leiostomus xanthurus*) ranging in standard length from 16 to 21 cm, and 4 skipjack tunas (*Katsuwonus pelamis*) whose fork lengths ranged from 44 to 50 cm were used for the mechanical tests and histological studies described below. The spots were obtained fresh from a local seafood market and the skin was either removed and studied at once or else the entire fish was frozen until its skin was needed. The tunas were obtained from the National Marine Fisheries Service Laboratory at Kewalo Basin, Honolulu, Hawaii; and transported frozen to North Carolina where the skin was removed from the frozen carcasses for testing.

Structure of the skin

For both species cryostat (American Optical, Model 830) sections 10 microns thick were made from fresh skin samples, mounted on slides with distilled water, and examined using a Leitz Ortholux polarized light microscope.

Mechanical testing

Uniaxial tensile stress tests were performed on skin samples in order to quantify stress-strain relationships in the direction of the collagen fibers and in both the longitudinal and hoop (circumferential) directions on the fish. Square or rectangular skin samples measuring several centimeters on a side were cut from the fish between the dorsal and ventral midlines and between the pectoral fins and the caudal peduncle. As much muscle was removed from the skin as possible using razor blades and scissors. Snap-swivels or alligator clips connected to fishing leader wire were attached to the edges of the samples for testing in the stress-strain machine described elsewhere (Wainwright *et al.*, 1978). To test for extension in the fibers themselves, skin samples were cut and stressed parallel to the previously determined fiber angle; during stressing, the orthogonal sides of the skin were allowed to deform freely. In a similar manner skin was cut and stressed uniaxially in the hoop and longitudinal directions.

An additional series of stress-strain tests was conducted by applying an increasing tensile load to the longitudinal axis while maintaining the hoop axis at a constant stress, and while monitoring strains of both the longitudinal and hoop axes simultaneously. The constant hoop stress applied in these tests was 0.12 MN/m^2, a value chosen to correspond with the more extensible lower range of the longitudinal stress-strain curves, and therefore a value likely to fall within the *in vivo* range of stresses occurring during normal swimming movements. The purpose of these biaxial stressing tests was to determine the relative contributions of the crossed-fiber system and the interfibrillar matrix material to the mechanical properties of the whole skin. If the extensibility of the skin is controlled solely by the helical fibers, the ratio of the hoop to longitudinal stresses applied is always equal to the tangent of the fiber angle resulting from these stresses times the tangent of the initial fiber angle. Using this relationship a longitudinal stress-strain curve can be constructed for any constant hoop stress applied, which will predict skin properties if they are due only to a set of continuous fibers. Comparison with the experimentally obtained stress-strain curve for whole skin should allow the roles of the fibers and the matrix to be assessed.

RESULTS

Structure of the skin

The skin of the Norfolk spot is typical of most teleosts, consisting of a covering of ctenoid scales that are anchored to the pigmented epidermis and are surrounded by a clear gelatinous material. Beneath the epidermis is the stratum compactum, or the collagenous layer of the skin. Microscopy reveals that alternating sheets of parallel fibers comprise this layer. Figure 1 is a polarized light micrograph of a section cut

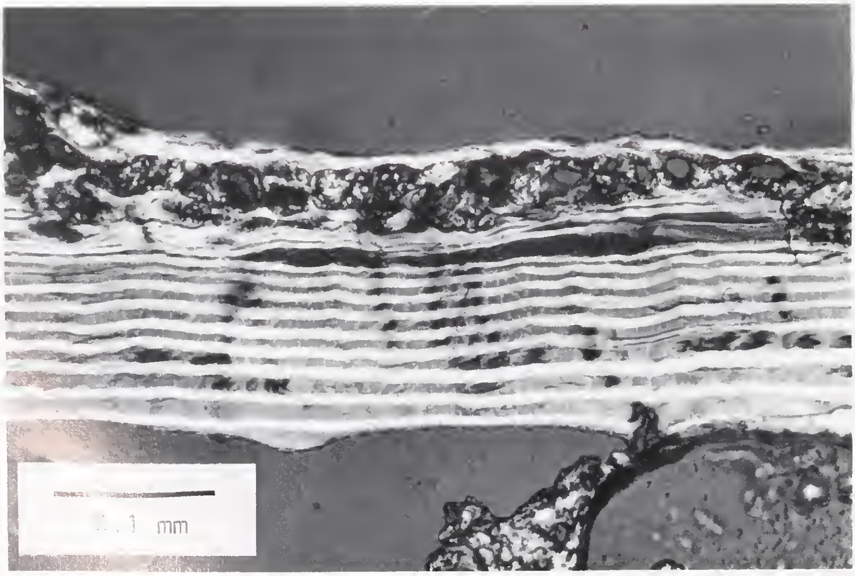

FIGURE 1. Polarized light micrograph of a radial section of spot skin cut parallel to one set of fibers; 12 fibers (or fiber layers) can be seen in long section.

perpendicular to the skin and parallel to one set of fibers; twelve fibers (one per layer) can be seen in long section. The fibers are generally smaller in diameter in the outermost layers of the skin, and range in thickness from about 4 to 10 μm. The total skin thickness for animals of this size class (about 20 cm standard length) examined ranged from about 220 to 300 μm.

Fiber angles were determined at numerous locations on the fish and those obtained from one specimen are shown in Figure 2. In general the angles of the forward leaning fibers were lower than those of the corresponding backward leaning fibers, with the former ranging from 45 to 62° and the latter from 62 to 80°. The fibers of the dorsal half of the caudal peduncle, however, were exceptional, with the forward leaning fibers forming an angle of 75° with the long axis of the fish and the backward leaning fibers forming an angle of only 30°. In all cases, the fiber angles of the forward leaning fibers were examined within a few degrees of the pitch of the scale rows that overlay the collagenous layer of the skin.

The skin of the skipjack tuna, like other tunas, differs from the skin of most teleosts in that it is devoid of scales over most of its surface. Scales are present only in an irregularly shaped region just behind the opercular opening; this scaled region is known as the "corselet." A thin layer of pigmented epidermis covers the rest of the fish, and this is easily abraided to reveal the fine collagen fibers below. The stratum compactum is only a thin, nearly transparent layer in this fish, and once the pigmented epidermis is abraided the axial musculature is readily visible through the collagenous layer.

Microscopy reveals that alternating sheets of parallel fibers comprise this layer, just as they do in the spot. Figure 3 is a polarized light micrograph of a section cut perpendicular to the skin and parallel to one set of fibers. Three fibers (one per layer) can be seen in long section and between these, fibers of the alternating three layers can be seen in oblique end section. The fibers near the upper and lower boundaries of the skin are the thinnest, about 20 μm in diameter, while those occupying the center of the skin's thickness are much thicker, about 80 μm in diameter. The total skin thickness for skipjack tunas of this size class (about 45 cm fork length) ranged from about 280 to 350 μm.

Fiber angles varied widely with respect to position on the fish, and like the spot, were generally not the same in the forward and backward leaning directions at the same point on the fish, as shown in Figure 4. In the middle regions of the fish fiber

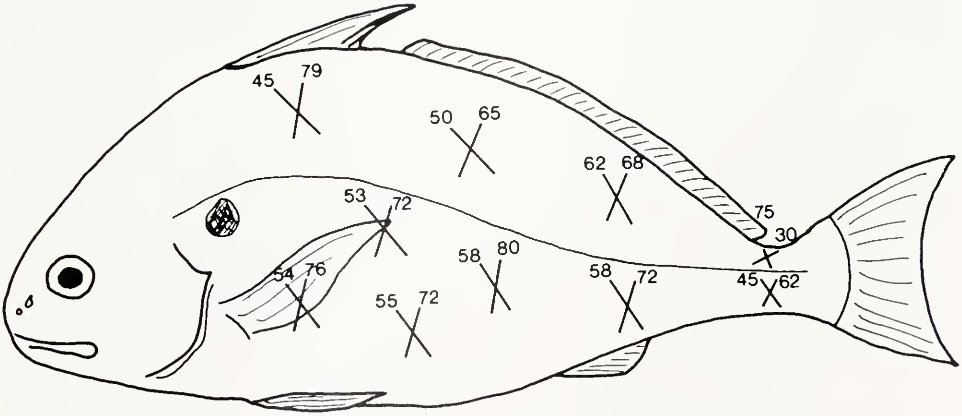

FIGURE 2. Fiber angles (in degrees) measured at various locations on one Norfolk spot specimen.

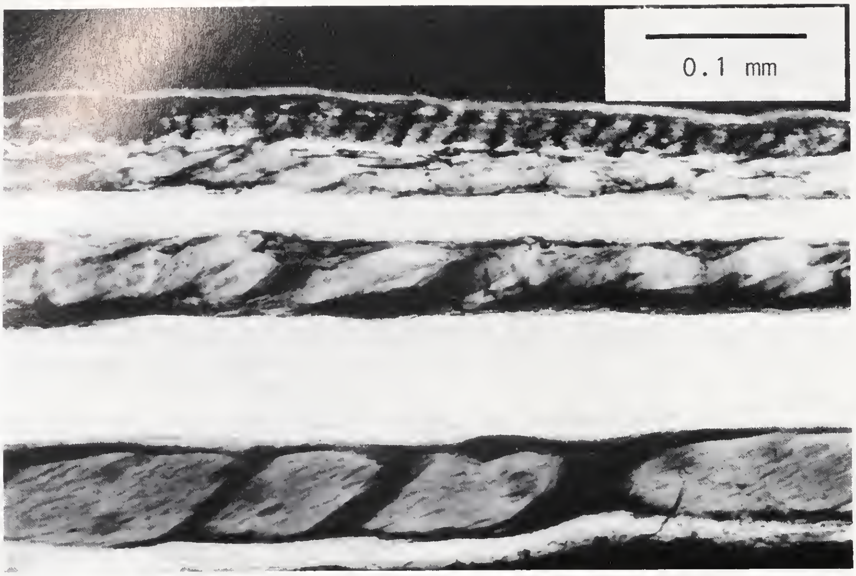

FIGURE 3. Polarized light micrograph of a radial section of skipjack tuna skin cut parallel to one set of fibers. Three fibers (or fiber layers) can be seen in long section alternating with three layers of fibers shown in oblique end section.

angles generally fell between 55 and 75°, but near the mid-dorsal and mid-ventral lines fiber angles became as low as 20° and as high as 87°. Fiber angles could be seen to change by several degrees as the mid-dorsal and mid-ventral lines were traversed and also changed slightly as the lateral line was traversed. In some cases fibers could be seen to curve as they suddenly changed pitch near the dorsal and ventral midlines.

Mechanical testing

In Figures 5 and 6 the results of uniaxial tensile tests are shown in the form of stress-strain curves for skin stretched in the direction of one set of fibers and in the hoop and longitudinal directions for each of the two species. For the skin of the Norfolk spot stretched in the direction of the fibers very low extensions are obtained

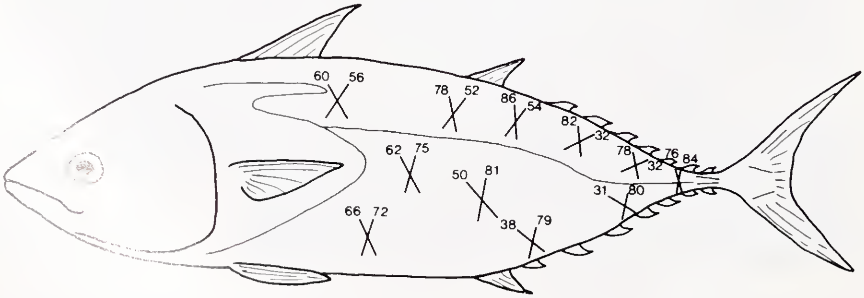

FIGURE 4. Fiber angles (in degrees) measured at several locations on one skipjack tuna specimen.

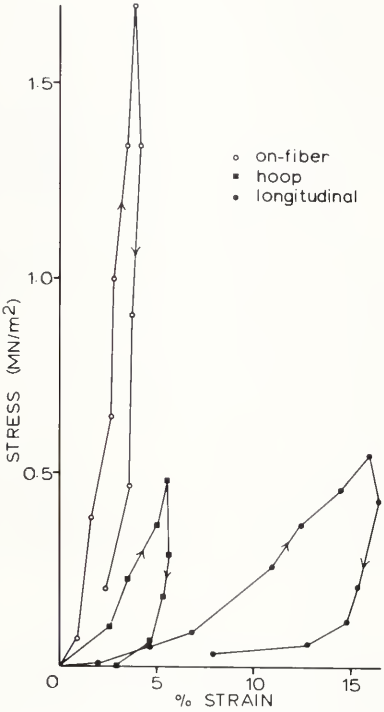

FIGURE 5. Typical stress-strain curves obtained for spot skin stretched uniaxially in the on-fiber, hoop, and longitudinal directions.

at high stresses. The fibers therefore appear to be reasonably inextensible, especially within the range of stresses applied to the hoop and longitudinal directions. The elastic modulus (the slope of the stress-strain curve in its steep region) obtained as an average of fifteen on-fiber pulls is 75.0 MN/m^2, as shown in Table I.

The stress-strain curves for skin of the spot subjected to uniaxial stretching in the hoop and longitudinal directions reveal anisotropy in the mechanical properties of the skin. Skin stressed in the hoop direction exhibits a J-shaped curve in which the skin extends by several percent while the stress remains low, then following this initial extension the stress-strain curve becomes steeper as the skin deforms less freely under the applied load. In contrast, skin stressed in the longitudinal direction exhibits a more linear stress-strain curve as it extends fairly uniformly over the range of stresses applied. The mean terminal elastic modulus for spot skin pulled in the longitudinal direction is 2.4 MN/m^2, which is significantly lower ($F_{(1,23)} = 14.4$, $P < 0.001$) than that of skin pulled in the hoop direction, a value of 16.4 MN/m^2. In addition, the skin is significantly stiffer in the on-fiber direction than in the hoop direction ($F_{(1,25)} = 13.9$, $P < 0.001$).

Like the skin of the spot, that of the skipjack tuna is relatively inextensible in the direction of the fibers, as shown in Figure 6. However, in contrast to the spot, skin of the tuna stressed in the hoop direction is about as stiff as skin stressed in the on-fiber direction. The mean elastic modulus of the skin stressed in the hoop direction is 60.2 MN/m^2, while that of skin stressed in the on-fiber direction is 36.2 MN/m^2, as shown in Table I, although these differences are not significant ($F_{(1,29)} = 1.96$, $P = 0.20$).

The skin of the tuna stressed uniaxially in the longitudinal direction is similar to

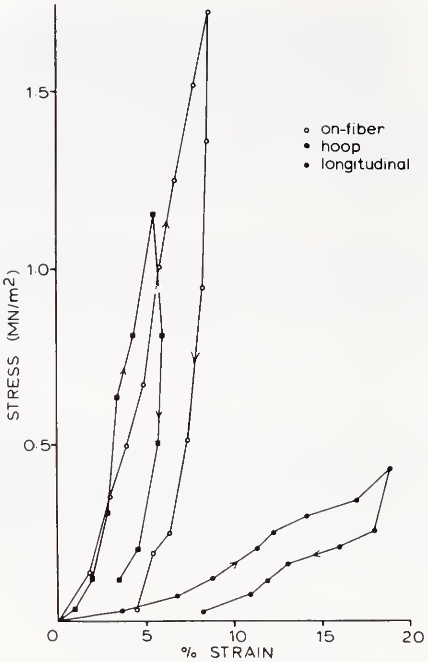

FIGURE 6. Typical stress-strain curves obtained for skipjack tuna skin stretched uniaxially in the on-fiber, hoop, and longitudinal directions.

that of the spot in that its stress-strain curve is more linear in shape and the skin is quite extensible. The differences in stiffness for skin stressed in the longitudinal direction compared to skin stressed in both the hoop and on-fiber directions are significant: for longitudinal *versus* hoop, $F_{(1,36)} = 17.16$, $P < 0.001$, and for longitudinal *versus* on-fiber, $F_{(1,29)} = 27.51$, $P < 0.001$. The mean elastic modulus for skipjack tuna skin stressed in the longitudinal direction is 6.9 MN/m², an order of magnitude lower than those of the other two directions tested.

TABLE I

Comparison of mean terminal elastic moduli for skin of the Norfolk spot and the skipjack tuna stressed uniaxially in three directions

	Norfolk spot elastic modulus	Skipjack tuna elastic modulus
On-fiber	7.50×10^7 N/m² (S.D. = 4.99×10^7) (n = 15)	3.62×10^7 N/m² (S.D. = 2.30×10^7) (n = 12)
Hoop	1.64×10^7 N/m² (S.D. = 0.63×10^7) (n = 12)	6.02×10^7 N/m² (S.D. = 5.44×10^7) (n = 19)
Longitudinal	2.41×10^6 N/m² (S.D. = 2.26×10^6) (n = 13)	6.92×10^6 N/m² (S.D. = 4.25×10^6) (n = 19)

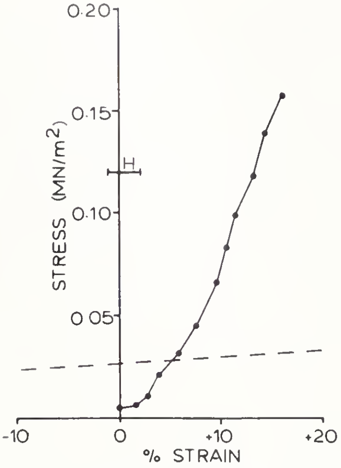

FIGURE 7. Biaxial stress-strain curve obtained for spot skin pulled in the longitudinal direction while the hoop direction was held at a constant stress of 0.12 MN/m^2. A theoretical curve for a pure fiber model subjected to the same ratio of stresses is shown as a dashed line. The bar labeled H shows the range of strains recorded in the hoop direction.

Breaking stresses and strains could not be determined for skin from either fish pulled in any direction. On application of high loads (approximately 50% higher than those shown in Figures 5 and 6 for each skin direction) the skin always failed at the clips attaching the skin to the testing device.

In general, skin samples pulled in each of the three directions did not return to

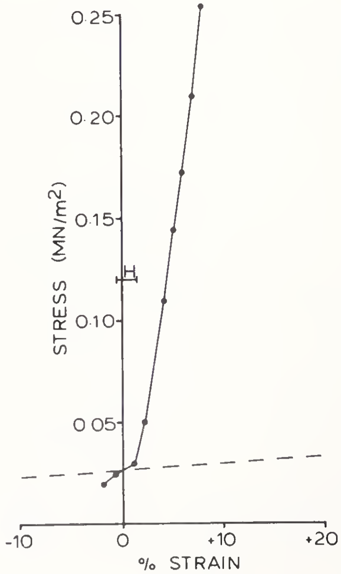

FIGURE 8. Biaxial stress-strain curve obtained for skipjack tuna skin subjected to the same conditions as in Figure 7.

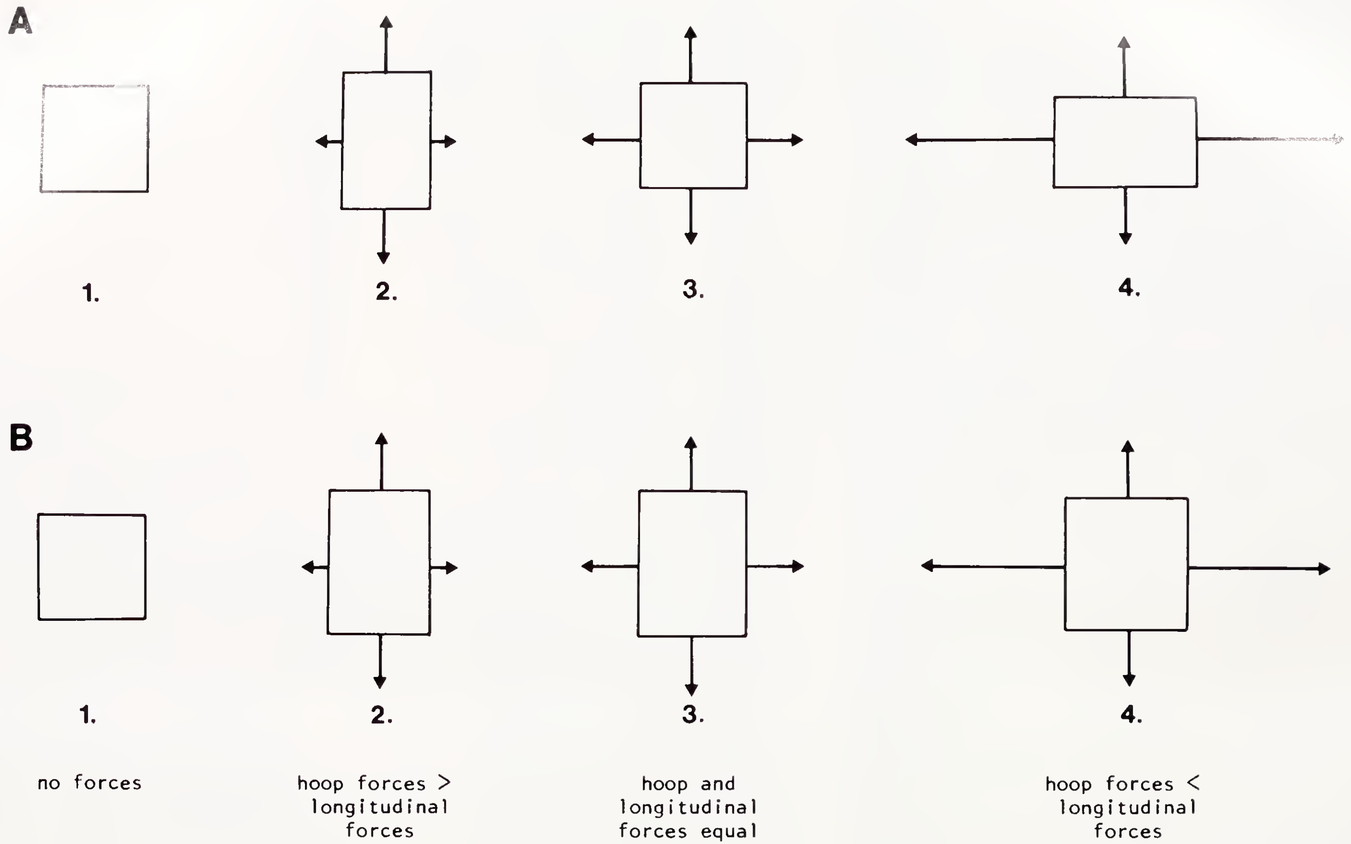
A
1.
2.
3.
4.
B
1.
2.
3.
4.
no forces
hoop forces > longitudinal forces
hoop and longitudinal forces equal
hoop forces < longitudinal forces

FIGURE 9. Schematic diagram of the behavior of (a) a simple crossed-fiber system, and (b) the skins of the spot and the tuna in biaxial stressing tests. The lengths of the arrows indicate the relative magnitudes of the forces applied to the hoop and longitudinal axes.

their original lengths once the load was removed, and the unloading curves always fell below the corresponding loading curves. However, this hysteresis may be exaggerated by frictional losses inherent in the testing device.

Biaxial stressing tests demonstrate that the skins of the spot and the skipjack do not behave as simple crossed-fiber systems. Stress-strain curves for tests performed in which the hoop axes were maintained at a constant stress of 0.12 MN/m^2 are shown for both fishes in Figures 7 and 8. Also shown in these figures are graphs of the predicted curve, based on the continuous fiber model described previously, subjected to the same ratio of stresses applied to the skin and having an initial fiber angle of 65°. It can be seen from these graphs that the skin of neither the spot nor the skipjack behaves in a manner similar to the model. Both are considerably stiffer than the model, having steeper curves than predicted, yet both require lower stresses applied to the longitudinal axes to achieve large initial extensions prior to crossing the predicted curve.

It is important to note that negative strains obtained for both the hoop and longitudinal directions were very small. Under these biaxial conditions much larger negative strains should have been observed, since the crossed-fiber system should allow both shrinking and stretching to occur simultaneously in the orthogonal sides of the skin. Instead, for both the spot and the skipjack, it appears that other material components within the skin dominate over the crossed-fiber system, as shown diagrammatically in Figure 9.

In this figure, row A depicts the behavior of a crossed-fiber model and row B depicts that of spot and tuna skin. When hoop forces are applied that exceed the longitudinal forces, the skin shown in B2 extends in the hoop direction but fails to contract in the longitudinal direction. In contrast, the model shown in A2 does contract in the longitudinal direction, and it therefore maintains a constant area. When longitudinal forces that equal the still-present hoop forces are applied to the model (A3), it extends in the longitudinal direction while contracting in the hoop direction. (Because forces are equal in both directions, it now has the same dimensions as it had in A1, before any forces were applied.) The skin shown in B3, however, does not contract in the hoop direction and extends very little in the longitudinal direction. (Its area has increased.) Much greater longitudinal forces are required (B4) to obtain both hoop contraction and longitudinal extension in the skin. The same longitudinal forces applied to the model (A4) result in greater degrees of both hoop contraction and longitudinal extension, with no increase in area.

Discussion

The collagenous layers within the skin of both the Norfolk spot and the skipjack tuna occupy the major part of the skin's thickness, and this relatively thick collagenous layer accounts for the skin's strong construction. In this respect the skins of these fishes are similar to those of both the eel and the shark, however, relative to total body size the spot and the tuna both have skin that is comparatively thin.

Mechanical tests of the skins of these fishes reveal other similarities and differences: while the terminal elastic moduli are similar in cases of uniaxial stressing in the hoop and longitudinal directions, the skin of the spot and skipjack both become stiffer at lower extensions than does eel skin. In the directions of the fibers themselves the skin is an order of magnitude less stiff than that of the eel, indicating that either the fibers themselves are not continuous over the lengths of the skin samples tested, or else the fine fibrils that comprise the fibers pull apart from each other when loaded directly along their axes.

More importantly, however, the skin of the spot and the skin of the skipjack do not undergo the shape changes expected of a crossed-fiber system, thus the skin is not capable of transmitting forces down the length of the fish. In a fish such as the eel or the shark, muscle contraction in the anterior region bends the fish, and so the skin on the convex side is extended in the longitudinal direction. This extension, however, produces contraction in the hoop direction as the fiber angle decreases, until these dimensional changes are resisted by pressurization of the body fluids beneath the skin. Now the skin becomes stiff, and further longitudinal force applied to the skin by the anterior muscles results in tension transmitted to the tail by the skin. In this way the skin of the eel or the shark can act as an external tendon, as suggested by Wainwright *et al.* (1978).

The results of biaxial tests of spot and skipjack skin reported here demonstrate that contraction of one side of the skin does not occur concomitant with extension of the orthogonal direction. Without this contraction tension cannot be transmitted by the skin down the length of the fish during swimming movements. Although the skin appears to have helically arrayed fibers suitable for an external tendon, it cannot function as one. Instead, for the spot and the skipjack, the crossed-fiber array of collagen seems to function primarily to keep the tough exterior surface of the fish smooth and free of kinks during swimming movements. A smooth surface is an important factor promoting hydrodynamic performance.

It is interesting to note that while eels and sharks possess skin capable of acting as an external tendon, this study suggests that teleosts in general probably do not. Eels and sharks share one feature of the skin that may relate to the ability to transmit forces: they have extremely thick skin. A thick skin is clearly beneficial to these fishes in consideration of some of their peculiar behaviors; some sharks have been observed to bite each other during courtship and eels spend much of their time burrowed beneath the substrate. In contrast, most teleosts have skin that is much thinner in proportion to their body size and with a reduction in skin thickness apparently comes a loss in the ability to transmit forces. Webb and Skadsen (1979) recently suggested that a reduction in skin mass is related to a fish's ability to accelerate rapidly during prey capture, and so it seems likely that those fishes that rely on this "stalk and sprint" method of feeding have skins with mechanical properties similar to the Norfolk spot and the skipjack tuna.

There is another morphological feature common to the eel and the shark but not to most advanced teleosts, and this is the arrangement of the axial musculature. Alexander (1969) described two patterns of the axial musculature of fishes, which he termed the "selachian" and "teleost" arrangements. Relevant to this study is the fact that the selachian pattern is found in the sharks, eels, *Amia, Acipenser,* and *Salmo,* while the teleost pattern is found in virtually all other teleosts that swim using lateral undulations of the body. In addition, Willemse (1972) noted that in the eel and the shark the myosepta are thickened at the periphery of the fish where they attach to the skin; such thickenings are not found in the spot or the tuna. Finally, those fishes having the selachian arrangement of the axial musculature tend to have relatively high vertebral numbers: eels and sharks have over 100 vertebrae, *Amia* has about 80, *Acipenser* has about 100, and *Salmo* has about 60. In contrast, tunas in general have about 35–40 vertebrae and the spot has only 24.

These similarities and differences suggest that perhaps those fishes in which the selachian muscle arrangement and high vertebral numbers are found also have skin capable of acting as an external tendon, although the functional relationships between these three components are unknown. In his functional analyses of the selachian and teleost muscle arrangements Alexander (1969) concludes that the selachian arrangement allows for the development of greater bending forces than does the teleost, but the teleost arrangement allows the fish to bend more quickly than the selachian. It is also important to recognize that anguilliform swimmers (eels and sharks) must transmit forces over a longer portion of the propulsive wave compared to the subcarangiform swimmers (*e.g.*, the spot) and the thunniform swimmers (tunas). It may prove possible, then, that fishes utilize one of two methods of swimming: contractions of the axial musculature, which bend the fish more slowly, do so in such a way as to generate forceful bending moments and the forces generated may be transmitted by the skin; or by contractions of the axial musculature that bend the fish more rapidly at the expense of weaker bending moments, and the forces developed by the muscles cannot be transmitted by the skin. Examination of the skins of *Amia, Acipenser,* and *Salmo,* as well as additional advanced teleosts, are needed before such positive correlations between muscle, skin, and backbone types can be established.

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THE CO_2 SENSITIVITY OF THE HEMOCYANINS AND ITS RELATIONSHIP TO Cl^- SENSITIVITY

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ABSTRACT

The effect of CO_2 on hemocyanin-oxygen binding is not generally related to the effect of Cl^-. Some hemocyanins respond to both and some to either one alone. The direction of the responses of O_2 affinity of the various hemocyanins to CO_2 is poorly correlated with the direction of responses to other effectors. The influence of CO_2 on *Busycon* and *Limulus* hemocyanins reaches its maximum at high pH. Since the effect can be abolished by restoring divalent cation activities to the control levels prior to the addition of CO_2, we suggest that the effect is not specific but rather indirect, by the pairing of the allosteric effectors Ca^{+2} and Mg^{+2} with the CO_2 anions. In contrast the effect of CO_2 on crustacean hemocyanins is greater at low pH and it can be enhanced by maintaining HCO_3^- levels within narrow limits and permitting PCO_2 to vary by a large factor. This finding suggests that the effective species is molecular CO_2. While Cl^- influences only oxygen affinity, CO_2 may influence cooperativity as well. In different species the effects of both Cl^- and CO_2 may or may not be great enough to be physiologically important.

INTRODUCTION

A site on the hemocyanin (Hc) molecule which is linked to the active site competitively binds divalent cations and H^+. Its molecular features and their physiological importance have been explored in detail (Arisaka and Van Holde, 1979; reviewed by Mangum, 1980; Miller and Van Holde, 1981).

The nature and significance of anion binding to the Hcs are less clear. While the effects of the organic anion L-lactate on the crustacean Hcs are beginning to be elucidated by a number of investigators (reviewed by Bridges and Morris, 1986), the influence of Cl^- is not as well understood. Cl^- specifically influences HcO_2 binding in the chelicerate arthropod *Limulus* (Sullivan *et al.*, 1974; Diefenbach and Mangum, 1983) and the gastropod mollusc *Busycon* (Mangum and Lykkeboe, 1979), but its effect on crustacean Hcs ranges from strong (Brouwer *et al.*, 1978) to absent (Truchot, 1975; Mason *et al.*, 1983). Brix and Torensma (1981) and Torensma and Brix (1981) concluded that the effects of Cl^- and CO_2 on gastropod Hcs are both allosteric and linked to one another, suggesting a Cl^--HCO_3^- site analogous to the divalent cation-H^+ site.

In fact, the effects of CO_2 in whatever form are even less clear than those of Cl^-. As pointed out earlier (Burnett and Infantino, 1984), only one of the several investigations on the subject convincingly demonstrates a significant and specific effect of CO_2 on O_2 affinity of the crustacean Hcs (Truchot, 1973). In contrast, a number of negative reports have appeared (reviewed by Burnett and Infantino, 1984; see also

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Morris and Bridges, 1985). After the paper by Burnett and Infantino (1984) was in press a large specific effect of CO_2 on a crab Hc was reported by Greenaway *et al.* (1983) but, curiously, the response is opposite to that found in *Carcinus* (Truchot, 1973).

An effect of very low levels of CO_2 on O_2 affinity of gastropod Hc has been found in three species (Mangum and Lykkeboe, 1979; Torensma and Brix, 1981) but the mechanism remains unclear. Mangum and Lykkeboe (1979) suggested that it may be either direct and specific, *viz.* by means of CO_2 binding to a site linked to the active site, or indirect and non-specific, *viz.* by means of ion-pair formation of HCO_3^- and CO_3^{-2} with the divalent cations, thus inhibiting the action of other allosteric effectors. Brix and Torensma (1981) and Torensma and Brix (1981) described the CO_2 effect as allosteric, but presented as supporting evidence only the fit of their data to the Monod-Wyman-Changeux model. However, the model, which describes a particular form of allosteric behavior, would not distinguish between direct and indirect allosteric actions.

We have investigated the effect of CO_2 and Cl^- on HcO_2 binding in a variety of species chosen because they are either the same as or closely related to those studied earlier and also because they may represent various combinations of CO_2 and Cl^- sensitivity. We have investigated the mechanisms of CO_2 effects on gastropod (and also chelicerate) Hcs, by controlling the levels of divalent cation activity while varying those of CO_2 anions. If the divalent cations are the immediate effectors, we would expect to see no effect of CO_2. We have investigated the mechanism of the effect on crustacean Hcs by controlling (in large part) the levels of the CO_2 anions while allowing CO_2 to vary by a large factor. In this case we would expect the CO_2 effect to vary if molecular CO_2 is the immediate effector.

MATERIALS AND METHODS

Collection and holding of animals

The crustaceans *Callinectes sapidus* Rathbun and *Palaeomonetes pugio* Holtuis, the chelicerate *Limulus polyphemus* (Linnaeus), and the gastropod mollusc *Busycon canaliculatum* Linnaeus were collected locally. The crustacean *Penaeus duorarum* Burkenroad was collected offshore of Beaufort, North Carolina and *Carcinus maenas* (Linnaeus) at Mt. Desert Island, Maine. The polyplacophoran mollusc *Cryptochiton stelleri* Middendorff was purchased from commercial sources. Animals were held at 16–25°C, depending on origin, and salinities (20–35‰) either identical to those at the collection sites or approximating those specified in previous investigations. The experimental temperature was chosen similarly.

Procurement and preparation of samples

If the ionic composition of the blood of a species was unknown it was determined with ion-selective electrodes (Mangum and Lykkeboe, 1979).

Blood samples were obtained by syringe sampling. The samples were centrifuged to remove debris, in the case of the arthropods after first declotting with a tissue grinder. The sera were then either used for O_2 binding measurements without modification or first dialyzed against the desired saline (4°C, 24 h).

O_2 binding

O_2 binding was determined by two methods: (1) for measurements in the presence of different levels of CO_2 a spectrophotometric method was employed (Burnett, 1979;

Burnett and Infantino, 1984). Mixtures of N_2 (99.9995%, scrubbed with Oxisorb), CO_2 (99.5%), and, depending on PO_2, either air (scrubbed with soda lime and Dri-Rite) or O_2 ([illegible]%) were prepared with Wosthoff pumps, humidified in a gas washing bottle, and passed over samples incubated in a thermostatically controlled shaker bath. The samples had been diluted by factors ranging from 13.5 to 101 with 0.05 *M* Tris Maleate buffered saline. Changes in absorbance (1 cm light path) were determined at 335–345 nm, depending on species, with a Bausch & Lomb Spectronic 20 colorimeter. These data are illustrated with open symbols.

(2) The cell respiration method (Mangum and Lykkeboe, 1979) was used to determine the effects of inorganic ions. Samples were diluted by 10% with buffered saline or buffered test solution (final concentration 0.05 *M* Tris Maleate buffer), and pH (Fisher Accumet with Ross electrode) and PCO_2 (Radiometer electrode, calibrated with 1.05 and 3.03% CO_2) were measured at the end of an experiment. These data are illustrated with closed symbols. In this procedure PO_2 is lowered at a constant rate by respiring yeast cells, which also excrete CO_2. To ascertain that PCO_2 in a buffered preparation does not change during an experiment, a second measurement was performed in parallel, but PCO_2 was measured instead of PO_2. Following equilibration of the electrode with the sample, no change was detected (Fig. 1).

O_2 carrying capacity

The total O_2 capacity of polyplacophoran blood is known from a single observation on a single individual (Redmond, 1962). After first subtracting the absorbance of deoxyHc to eliminate light scatter, an estimate was made from the spectrophotometric data using the extinction coefficient for gastropod Hc (Nickerson and Van Holde, 1970). Total O_2 was also obtained from the records made during the cell respiration procedure as follows (Fig. 2): The area under the (dashed) line representing a constant rate of O_2 depletion describes the volume of free O_2, which can be evaluated knowing

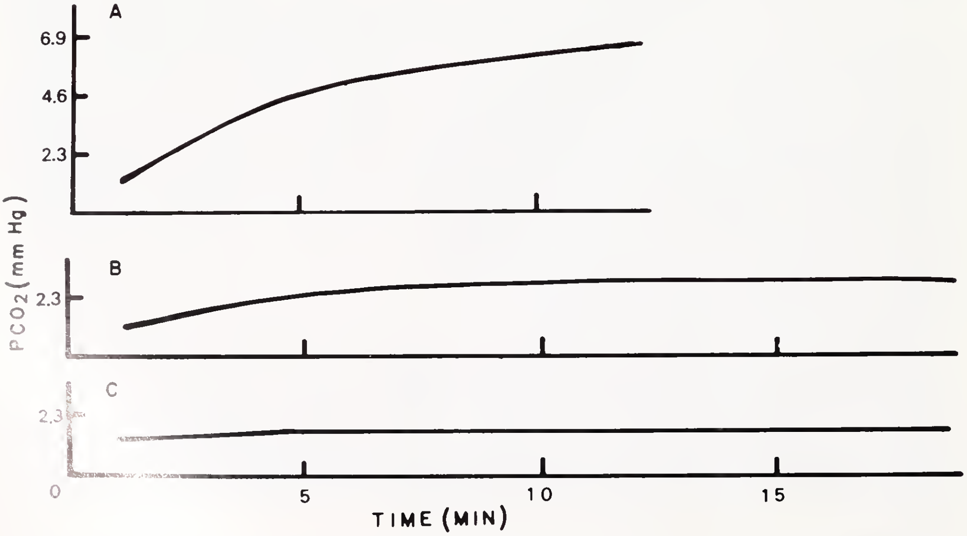

FIGURE 1. Recording of PCO_2 during depletion of O_2 in: A. Saline, with no Hc and no exogenous buffer. B. Saline + *Penaeus duorarum* Hc but no exogenous buffer. C. Saline + *P. duorarum* Hc and 0.05 *M* Tris Maleate buffer (pH 7.6). 20°C.

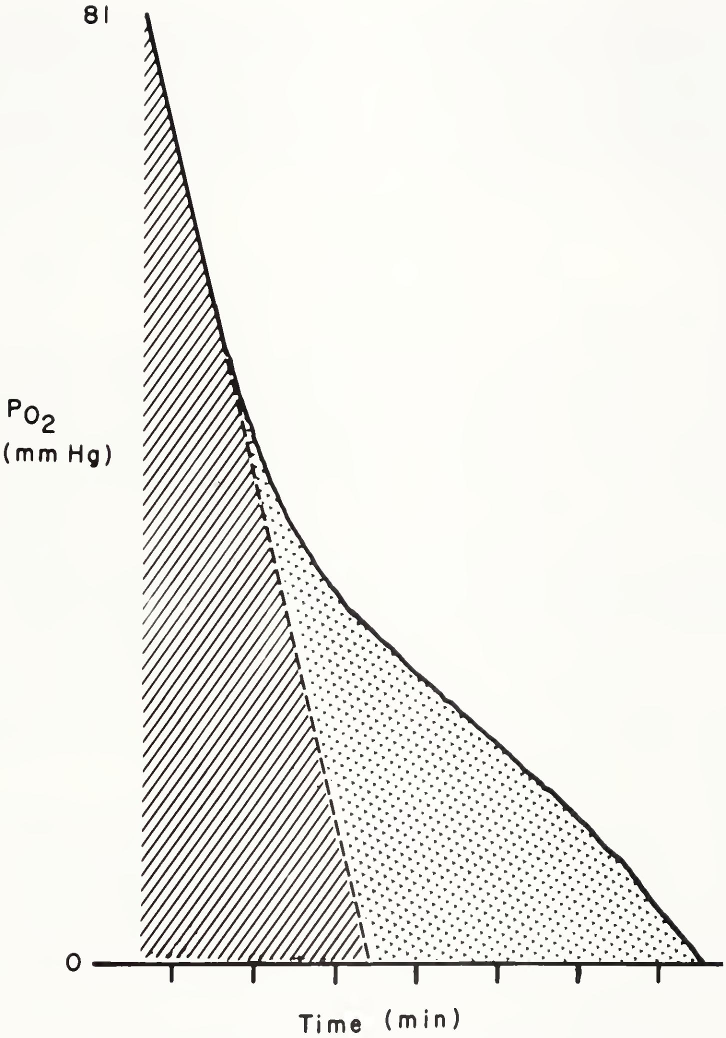

FIGURE 2. O_2 depletion by yeast cells in presence of *Cryptochiton stelleri* Hc. pH 7.43, 15°C. Hatched area shows volume of free O_2; stippled area shows volume of HcO_2.

temperature and solute concentration. The area under the curve representing the deviation from that line describes the volume of Hc-bound O_2, which can be evaluated as a fraction or, more often, a multiple of the free O_2 area.

Data analysis

When pH, CO_2, and Cl^- were either controlled or when all three had no detectable effect, the significance of differences between mean values of P_{50} or n_{50} was estimated by Student's *t*-test; the probability of a significant difference is specified below. When pH and CO_2 were varied simultaneously and Cl^- controlled, the data were described by semilogarithmic (log Y) regression lines and the overlap (if any) between 95% confidence intervals around the lines used as the criterion of significance. When NaCl was varied while pH and PCO_2 were controlled, the P_{50} data were described by semilogarithmic regression lines and the difference of the slope from zero (at $P = .05$) used as the criterion. Finally, when HCO_3^- and NaCl were controlled while pH and PCO_2 were varied simultaneously, the data were described by semilogarithmic regression lines and the overlap (if any) between 95% confidence intervals around the slopes was used as the criterion. If a substance is said below to have an effect these criteria were exceeded.

RESULTS

Busycon canaliculatum

Mangum and Lykkeboe (1979) showed that in the channeled conch Cl^- has a specific effect on HcO_2 binding and that, at high pH, the addition of molecular CO_2 lowers the oxygen affinity of low salinity samples and raises the oxygen affinity of high salinity samples, but only above pH 7.9 where the reverse Bohr shift is quenched by divalent cations. They suggested that, if the CO_2 effect were indirect (*viz.* by means of ion-pair formation with the divalent cations), the difference between high and low salinity could be explained quantitatively by their findings on the effects of individual inorganic ions: at high salinity the addition of CO_2 immobilizes some of the divalent cations that normally lower oxygen affinity but enough Ca^{+2} and Mg^{+2} remains to quench the reverse Bohr shift; the net result is an increase in oxygen affinity. At low salinity, where Ca^{+2} and Mg^{+2} are already scarce, their immobilization by CO_2 does not leave enough free divalent cations to quench the reverse Bohr shift. Na^+ and Cl^- become the dominant inorganic ions, and they lower oxygen affinity, which is in fact the net result. The present findings confirm the effect of CO_2 at high salinity, only above about pH 7.9 (Fig. 3).

To more directly demonstrate the mechanism the following experiment was performed: first a sample of serum was dialyzed against buffered physiological saline for 24 h and O_2 binding determined. Then 25 m*M* $NaHCO_3^-$ was added to the stirred preparation in which a Ca^{+2} selective electrode was immersed, and the change in Ca activity noted. O_2 binding was determined again. While the sample was stirred with the Ca electrode immersed, $Ca(NO_3)_2$ was then added in sufficient quantity to restore the original level of Ca activity; O_2 binding was measured a third time. This experiment controls for pair-formation of the CO_2 anions with Ca^{+2} but not Mg^{+2}. It could not be performed with a total divalent cation selective electrode for a variety of reasons, the most important of which are the lower sensitivity and resolution of the electrode. Normally these problems are mitigated by chelating Ca with EGTA and then measuring pMg alone; the remedy would not have been possible in the present context without eliminating an important effector of O_2 binding. Since the percent pair formation of Ca and Mg with the CO_2 anions is the same (Kester and Pytkowicz, 1969) and since the effects of Ca^{+2} and Mg^{+2} on O_2 binding are very nearly so (Mangum and Lykkeboe, 1979), the pair formation with Mg was calculated and additional $Ca(NO_3)_2$ was added to simulate the original Mg^{+2} activity using Ca^{+2} instead. Changes in monovalent anions of those small magnitudes have no detectable effect (Mangum and Lykkeboe, 1979). O_2 binding was determined a fourth time.

As reported earlier (Mangum and Lykkeboe, 1979), the addition of $NaHCO_3$ to the blood raises the O_2 affinity of high salinity blood and lowers the O_2 affinity of low salinity blood (Table I). It does not clearly influence cooperativity ($P = .09–0.70$). The third and fourth steps of the experiment were performed on high salinity blood. When the original activity of only Ca was restored, O_2 affinity dropped slightly though sig-

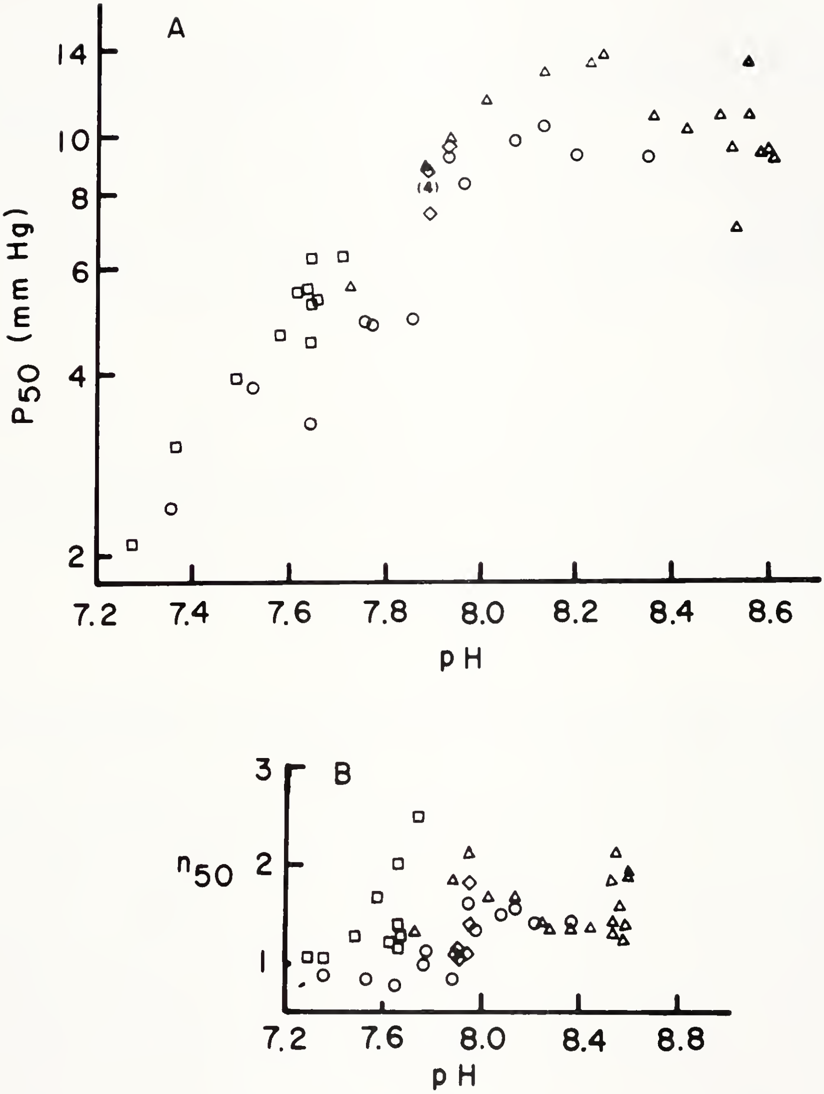

FIGURE 3. Effect of CO_2 on *Busycon canaliculatum* HcO_2 binding. Tonometric method. (○) $PCO_2 = 0$; (△) $PCO_2 = 0.74$ mm Hg; (◇) $PCO_2 = 7.4$ mm Hg; (□) $PCO_2 = 14.8$ mm Hg. 20°C. Serum was diluted with 0.05 *M* Tris maleate buffered high salinity saline (from Mangum and Lykkeboe, 1979). A. Oxygen affinity. B. Cooperativity.

nificantly, but still differed from the control value. When additional Ca was added to simulate the change in total divalent cations, O_2 affinity decreased further to a value that is indistinguishable from the control.

Cryptochiton stelleri

The Hc of the giant sea cradle is clearly cooperative and it has a small (though significant) normal Bohr shift (Fig. 4). The present results essentially agree with earlier

TABLE I

Effect of NaHCO$_3$ and Ca(NO$_3$)$_2$ on Busycon canalicalatum HcO$_2$ binding[1]

	HS		LS	
	P_{50}	n_{50}	P_{50}	n_{50}
Control, PCO_2 1.50–1.59 mm Hg	10.75 ± 0.31 (8)	1.31 ± 0.04 (8)	9.70 ± 0.40 (6)	1.36 ± 0.13 (6)
+ 25 m*M* $NaHCO_3$ PCO_2 5.5–5.7 mm Hg	9.31 ± 0.17 (6)[2]	1.40 ± 0.03 (6)	10.70 ± 0.30 (8)	1.43 ± 0.25 (8)
Same, + Ca^{+2} to Equal pCa in control. PCO_2 5.7 mm Hg	9.95 ± 0.20 (9)[3]	1.33 ± 0.05 (9)	—	—
Same, + Ca^{+2} to Equal pCa + pMg in control	10.72 ± 0.20 (8)[4]	1.33 ± 0.06 (8)	—	—

[1] Cell respiration method:serum was dialyzed against 0.05 *M* Tris maleate buffered high (HS) or low (LS) salinity saline (from Mangum and Lykkeboe, 1979). 19.7–20.1°C, pH 8.18–8.23. Mean ± S.E. (N).

[2] $P = 0.0035$ *vs* control.

[3] $P = 0.025$ *vs* control, 0.0375 *vs* + $NaHCO_3$ alone.

[4] $P = 0.04$ *vs* + Ca^{+2} to equal pCa in control, 0.97 *vs* control.

findings (Manwell, 1958), although the comparison is made somewhat difficult by the different pH ranges investigated. The slope of a regression line describing the two sets of control data for P_{50} in Figure 4A is −0.20 ± 0.04 (95% C. I.). Also in the combined control data, there is a small but significant increase in cooperativity with pH.

The O_2 affinity data for 0 and 14.8 mm Hg PCO_2 differ significantly in the pH range 7.2–7.6 but not in the range 7.7–7.85 (Fig. 4A). At high PCO_2 cooperativity is lower ($P = 0.002$) and there is a small but significant decrease with pH. Neither HcO_2 affinity (Fig. 4B) nor cooperativity (1.87 ± 0.09 S. E., N = 6) respond to NaCl.

The data in Figure 4 were obtained using blood taken from the hemocoel directly into a hypodermic needle passed through two adjacent dorsal shell plates. When an initial attempt to perform the operation was unsuccessful, a blood sample was obtained by slitting the foot and draining the hemocoel. No other organs were damaged although some mucus was produced. The sample formed a blue precipitate and, after centrifugation at low speed, the supernatant fluid did not combine reversibly with oxygen. When the pellet was washed with saline, it did not go into solution but it still combined reversibly with oxygen. Measured with the cell respiration technique, oxygen affinity was higher than that of the syringe sample, but only by about 25%; cooperativity was lower but still easily detected (n_{50} = 1.72 ± 0.19 S. E., N = 12). Apparently the native higher order structure is not critical to cooperativity and a relatively low O_2 affinity.

The difference in absorbance between oxygenated and deoxygenated samples, corrected for the dilution factor indicates that the native Hc concentration in one animal was about 0.74 g/100 ml, yielding an HcO_2 capacity of only about 0.33 (total O_2 capacity = 0.91) ml/100 ml. The integrals obtained from the records made during the cell respiration procedure indicate an HcO_2 carrying capacity in another animal of 0.22 ml/100 ml or a total blood O_2 carrying capacity of 0.80 ml/100 ml. The results support the inference of an extremely low O_2 carrying capacity of the blood in this class (Redmond, 1962).

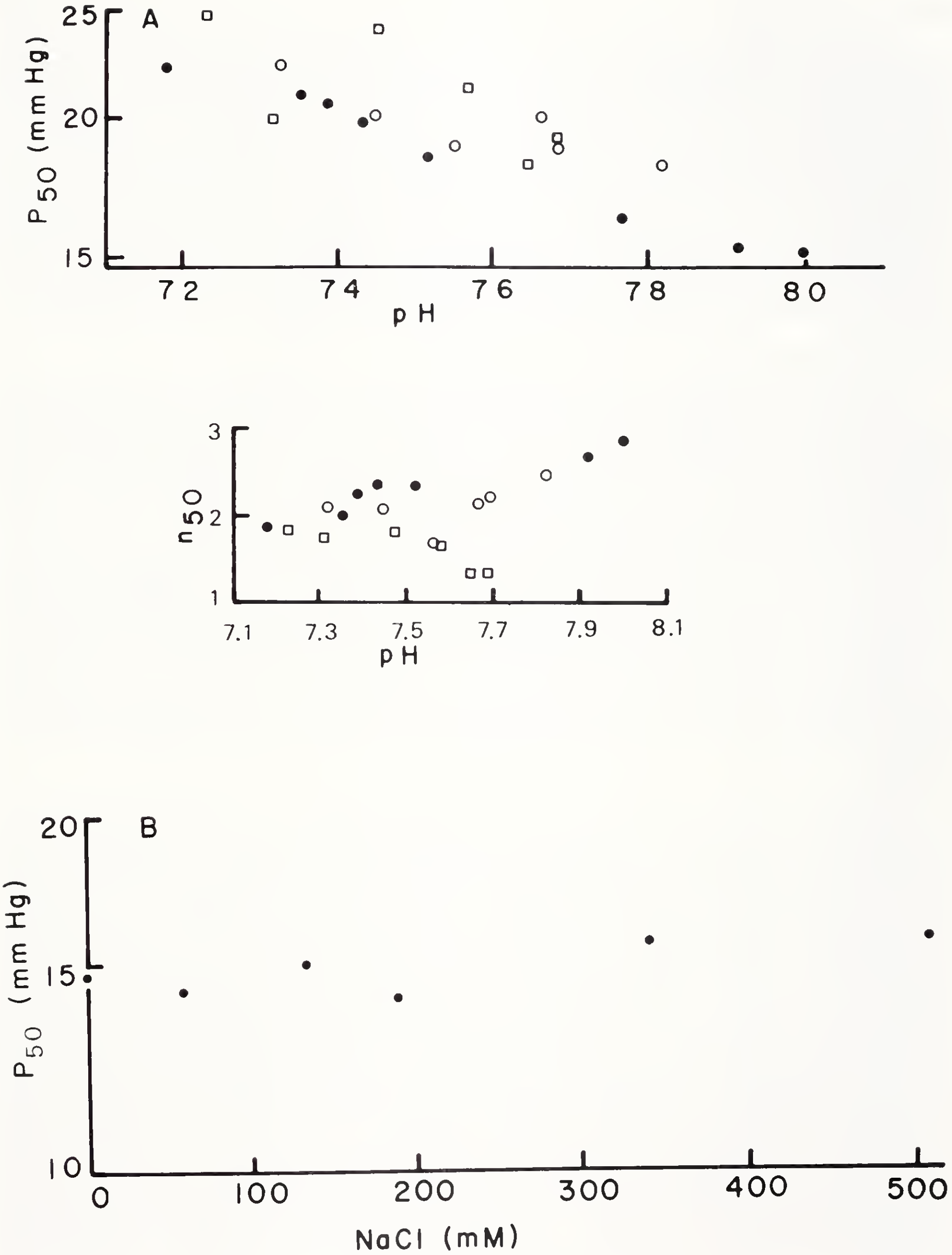

FIGURE 4. A. Effect of CO_2 on *Cryptochiton stelleri* HcO_2 binding. Tonometric method: serum was diluted with 0.05 *M* Tris maleate buffered, filtered seawater (35‰) in which the animals had been shipped. (○) $PCO_2 = 0$, (□) $PCO_2 = 14.8$ mm Hg. 15°C. Cell respiration method: after dialysis against seawater, Tris maleate buffer (final concentration 0.05 *M*) was added to the serum. (●) PCO_2 1.32–5.41 mm Hg. B. Effect of NaCl on HcO_2 affinity. Cell respiration method: serum was dialyzed against 10 m*M* $Ca(NO_3)_2$ + 0.05 *M* Tris maleate buffer, pH 7.62–7.65. PCO_2 3.0–3.3 mm Hg. 15°C.

Penaeus duorarum

At pH 7.6, which is likely to approximate the physiological value, the Hc of the pink shrimp has a moderate oxygen affinity and pronounced cooperativity. In the pH range studied it also has a large normal Bohr shift (Fig. 5); the slope of a regression line describing the two sets of control data is −1.09 ± 0.19 (95% C. I.). The data are virtually indistinguishable from those reported earlier for *P. setiferus* (Brouwer *et al.*, 1978). As also in *P. setiferus* (Brouwer *et al.*, 1978), *P. duorarum* HcO_2 affinity clearly responds to NaCl (Fig. 5B). However, it responds identically to $NaNO_3$, Na_2SO_4 (plotted as m*M* cation), and choline Cl^-. There is a significant decrease in cooperativity ($P = 0.05$–0.001, Fig. 5B). Neither property responds to CO_2, although the trend in cooperativity is the same as that in the data for CO_2 sensitive Hcs ($P = 0.15$, Fig. 5A).

Palaeomonetes pugio

At putative physiological pH the Hc of the grass shrimp has an extremely low O_2 affinity and fairly little cooperativity (Fig. 6). The slope of a semilogarithmic regression line describing the control data for P_{50} is −1.33 (±0.56 95% C. I.). HcO_2 affinity clearly responds to CO_2 (Fig. 6A), which does not appear to influence cooperativity ($P = 0.18$). The pH dependence of P_{50} at high PCO_2 appears to be about the same as that at low PCO_2, but the estimate made by regression analysis is much greater (−1.75 ± 0.11). In view of the far greater variability in the control data ($r^2 = 0.756$) the numerical estimate of the Bohr shift at high PCO_2 ($r^2 = 0.998$), which shows extremely great pH dependence, is probably the more accurate of the two. NaCl clearly raises HcO_2 affinity but does not change cooperativity (Fig. 6B). Due to the great difficulty of obtaining ample volumes of blood from these always small animals, which were even smaller than usual at the time of the experiment on NaCl sensitivity because the population had just reproduced, we did not investigate the specificity of this response.

Callinectes sapidus

The Hc of the blue crab does not respond specifically to Cl^- (Mason *et al.*, 1983). Oxygen affinity (but not cooperativity) does respond to CO_2 (Fig. 7). Because it was concluded earlier that the Hc of a species belonging to the same genus (*C. bellicosus*) is not sensitive to CO_2 within the range 1.5–7.4 mm Hg, the response of *C. sapidus* was examined in more detail. While there may appear to be little or no difference in P_{50} at 0 and 1.5 mm Hg (Fig. 7), the two sets of data are in fact significantly different. When the data for *C. bellicosus* are analyzed similarly, however, an effect of CO_2 can be demonstrated only at 23°C and only at pH 7.5 and above (Burnett and Infantino, 1984). Of the other four species examined by Burnett and Infantino (1984) a similar trend may be present in the data for *Pachygrapsus crassipes* (though $P > .05$); however, no sign of an effect can be perceived in the remaining three (Burnett and Infantino, 1984). Thus we suggest that our original conclusion, *viz.* that CO_2 is not an important effector of HCO_2 binding in the five species, was essentially correct (Burnett and Infantino, 1984). Regardless, only at much higher PCO_2 is there an appreciable response of HCO_2 affinity in *C. sapidus;* cooperativity does not respond even at that level (Fig. 7).

In an attempt to identify the CO_2 species responsible for the effect, we dialyzed aliquots of a sample against either (1) saline containing no exogenous CO_2, or (2) saline containing high levels of $NaHCO_3$. When pH was adjusted by adding Tris maleate buffered saline (final concentration 0.05 *M*), PCO_2 varied by more than an order of magnitude while HCO_3^- varied by only about 25% (Fig. 7B). The oxygen affinity of this sample was depressed even more at low pH and high PCO_3 than at high pH and low PCO_2. If this result were due to the greater change in pCa at high

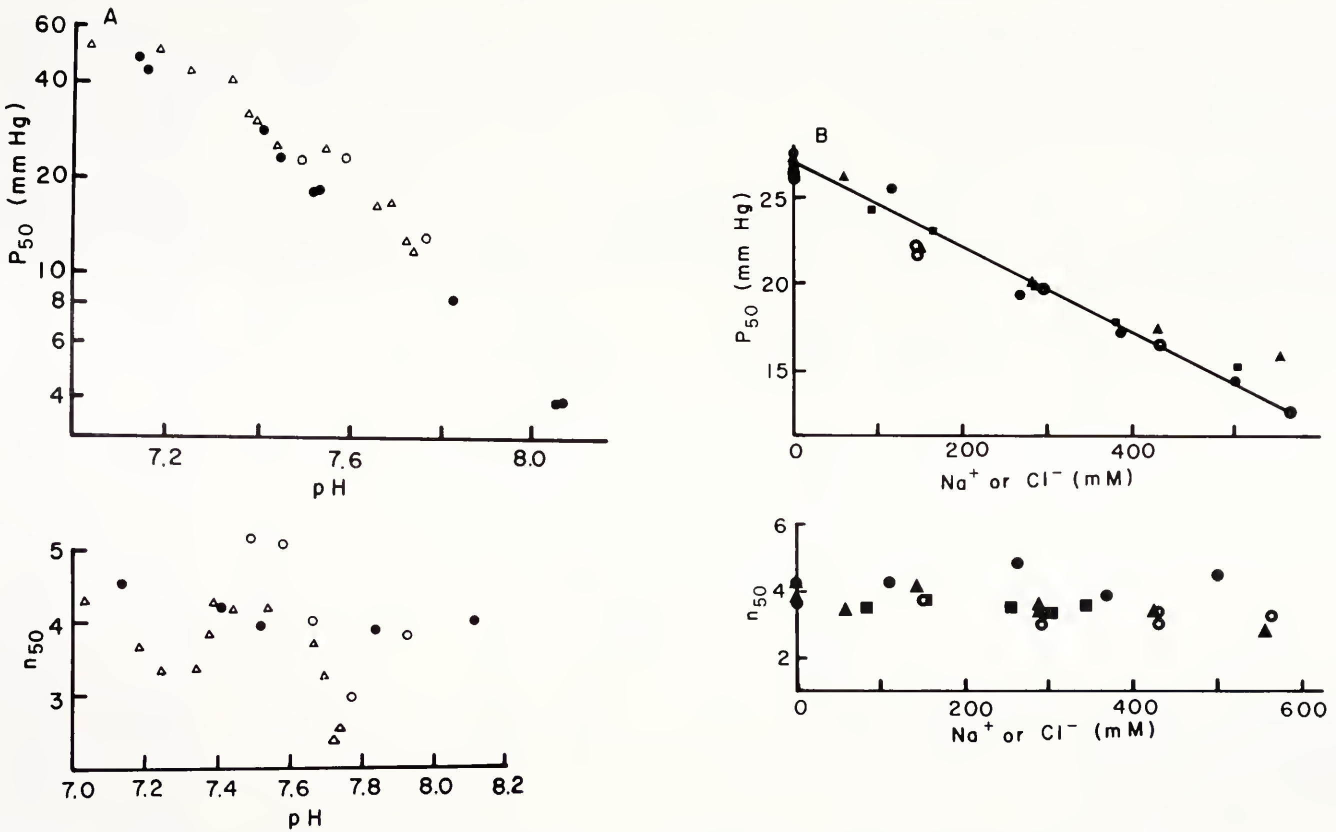

FIGURE 5. A. Effect of CO_2 on *Penaeus duorarum* HcO_2 binding. Tonometric method: serum was diluted with 0.05 *M* Tris maleate buffered saline containing 11 m*M* KCl, 14 m*M* $CaCl_2$, 39 m*M* $MgCl_2$, 433 m*M* NaCl, and 32 m*M* Na_2SO_4. (○) $PCO_2 = 0$, (△) $PCO_2 = 14.8$ mm Hg. Cell respiration method: serum was dialyzed against buffered saline described above, $PCO_2 = 1.30–6.60$ mm Hg. B. Effect of (●) NaCl, (■) $NaNO_3$, (▲) Na_2SO_4, and (○) choline Cl^-. Cell respiration method: serum was dialyzed against 0.05 *M* Tris maleate buffered $Ca(NO_3)_2$ (15 m*M*, pH 7.58–7.69, $PCO_2 = 2.0–3.3$ mm Hg). 20°C.

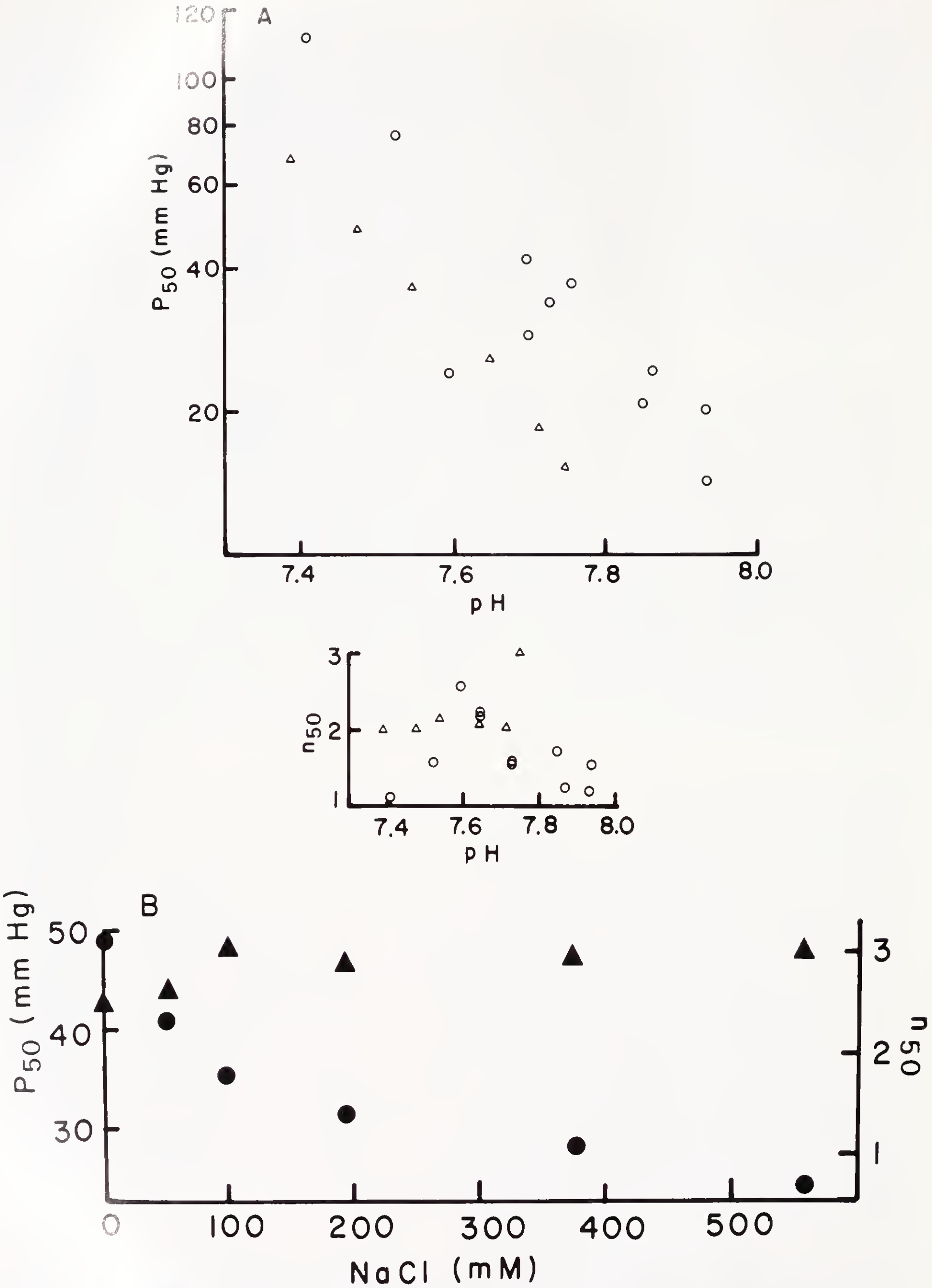

FIGURE [illegible]. A. Effect of CO_2 on *Palaemonetes pugio* HcO_2 binding. Tonometric method: serum was dialyzed against saline containing 261 m*M* NaCl, 8.1 m*M* KCl, 6.3 m*M* $CaCl_2$, 4.4 m*M* $MgCl_2$, and 29.5 m*M* Na_2SO_4 (from Mantel and Farmer, 1983), and diluted with 0.05 *M* Tris maleate buffered saline. (○) $PCO_2 = 0$, (△) $PCO_2 = 14.8$ mm Hg. 17°C. B. Effect of NaCl on HcO_2 binding. Cell respiration method: serum was dialyzed against 10 m*M* $Ca(NO_3)_2$ + 0.05 m*M* Tris maleate buffer, pH 7.65–7.68, PCO_2 1.3–1.5 mm Hg. 25°C. (●) P_{50}. (▲) n_{50}.

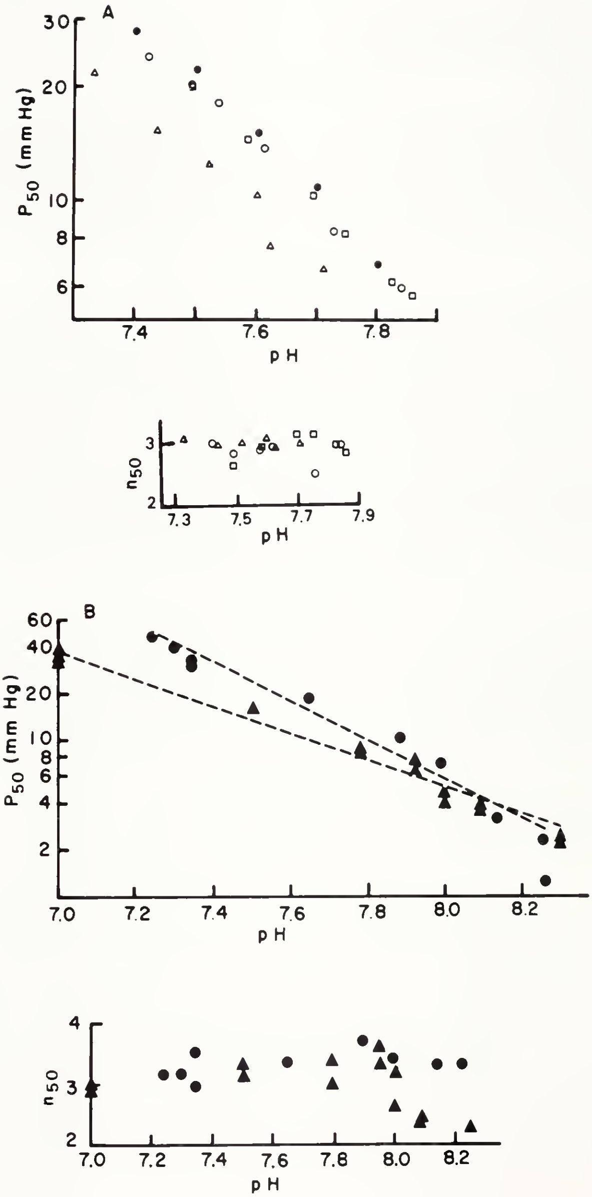

FIGURE 7. A. Effect of CO_2 on *Callinectes sapidus* HcO_2 binding. Tonometric method: serum was diluted with 0.5 *M* Tris maleate buffered high salinity saline (from Mason *et al.*, 1983). 25°C. (○) $PCO_2 = 0$, (□) $PCO_2 = 1.5$ mm Hg; (△) $PCO_2 = 14.8$ mm Hg. (●) For comparison, unpublished data collected earlier by cell respiration method: serum was dialyzed against same Tris maleate buffered saline. 25°C, PCO_2 unknown. B. Effect of high $NaHCO_3$. Cell respiration method: in controls (●), PCO_2 and $[NaHCO_3]$ vary from 0.32 mm Hg and 4.09 m*M* at pH 8.25 to 6.98 mm Hg and 1.69 m*M* at pH 7.24. In experiments (▲), PCO_2 and $[NaHCO_3]$ vary from 1.2 mm Hg and 13.7 m*M* at pH 8.25 to 1.4 mm Hg and 10.61 m*M* at pH 7.00. 25°C. Dashed lines, which differ significantly, were fitted by regression analysis.

than at low pH. O_2 affinity should have been lowered at high pH and unchanged at low pH rather than the other way around.

Carcinus maenas

Truchot (1973, 1975) showed earlier that, in European members of this species, Cl^- has no effect whereas CO_2 clearly raises O_2 affinity. Our data confirm the CO_2 effect, which occurs in about the same magnitude in the North American population (Fig. 8). They also show that CO_2 significantly lowers cooperativity and its pH dependence.

Limulus polyphemus

Cl^- has a specific effect on HcO_2 affinity (but not cooperativity) in the horseshoe crab (Sullivan *et al.*, 1974; Diefenbach and Mangum, 1983). CO_2 has a significant effect on O_2 affinity as well; an apparent decrease in cooperativity is not quite significant ($P = 0.11$; Fig. 9). The experiment designed to identify the mechanism of the CO_2 effect on *Busycon* Hc was also performed on *Limulus* Hc, with the exception that the step simulating the effect of changing only pCa was omitted. The addition of $NaHCO_3$ significantly raises O_2 affinity (Table II) but not cooperativity ($P = 0.19$) and the addition of $Ca(NO_3)_2$ to restore the original levels of free Ca^{+2} and Mg^{+2} lowers O_2 affinity back again to a value that does not differ from the control.

DISCUSSION

Various combinations of Cl^- and CO_2 sensitivity of the Hcs can be found. In any particular species HcO_2 affinity may be sensitive to both (*e.g.*, *Busycon, Limulus,* and *Palaeomonetes*), to Cl^- but not CO_2 (*Penaeus*) or to CO_2 but not Cl^- (*Cryptochiton,*

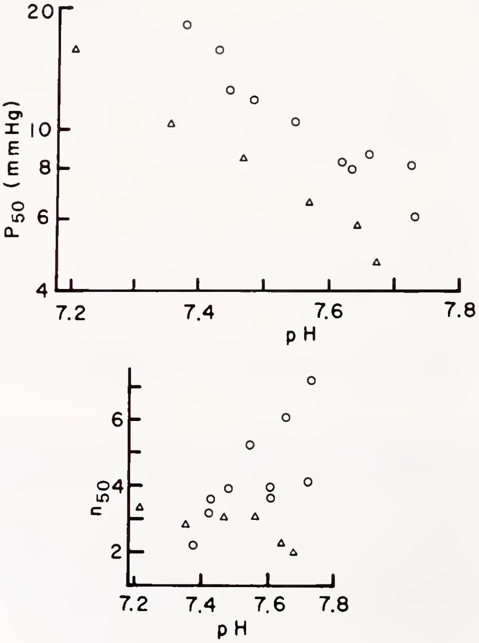

FIGURE 8. Effect of CO_2 on *Carcinus maenas* HcO_2 binding. Tonometric method: serum was diluted with 0.05 *M* Tris maleate buffered saline (from Robertson, 1960). 16°C, (○) $PCO_2 = 0$, (△) $PCO_2 = 14.8$ mm Hg.

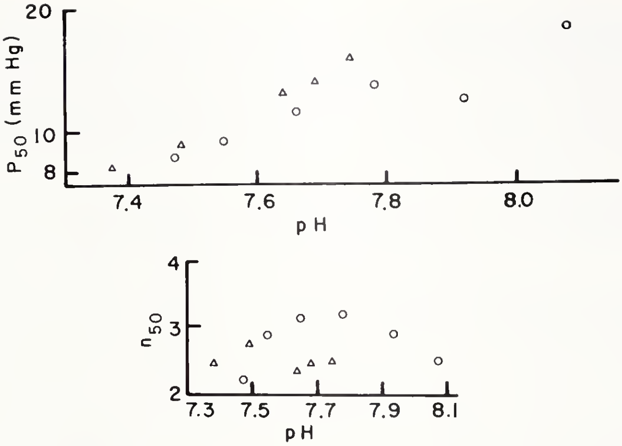

FIGURE 9. Effect of CO_2 on *Limulus polyphemus* HcO_2 binding. Tonometric method: serum was diluted with 0.05 *M* Tris maleate buffered high salinity saline (from Towle and Mangum, 1982). (○) $PCO_2 = 0$, (△) $PCO_2 = 14.8$ mm Hg. 25°C.

Carcinus, and *Callinectes*). In contrast the effect of CO_2 (if it occurs) is poorly correlated with sensitivity to other ionic effectors. While CO_2 consistently raises the O_2 affinity of Hcs (*Busycon, Limulus*) with reverse Bohr shifts (and, more importantly, positive divalent cation responses), it may either raise (*Callinectes, Carcinus*, and *Palaeomonetes*), lower (*Cryptochiton*) or have no effect on (*Penaeus*) the O_2 affinity of Hcs with normal Bohr shifts. Different combinations of CO_2 sensitivity of HcO_2 affinity and cooperativity can also be found. CO_2 may affect O_2 affinity but not cooperativity (*e.g., Busycon, Limulus*, and *Callinectes*), both (*Carcinus, Cryptochiton*, and perhaps *Palaeomonetes*) or perhaps neither (*Penaeus*). If CO_2 does have an effect, it generally lowers cooperativity and reduces its pH dependence.

Our results suggest that the chemical species responsible for the effect of CO_2 on portunid crab HcO_2 affinity is molecular carbon dioxide. The effect is greater at low than at high pH and the effector changes O_2 affinity in the same direction as the divalent cations. We can think of no indirect mechanism that would produce such a response. Moreover, the experiment in which HCO_3^- was held within a relatively narrow range while PCO_2 was allowed to change by a large factor produced a much greater effect of CO_2 at low pH, also implicating the molecular species.

Our results also support the hypothesis that the mechanism responsible for the effect on *Busycon* and *Limulus* Hcs is the action of the CO_2 anions on the allosteric effectors free Ca^{+2} and Mg^{+2} rather than a direct effect. It occurs only at high pH, it

TABLE II

Effect of $NaHCO_3$ and $Ca(NO_3)_2$ on Limulus polyphemus *HcO_2 binding*[1]

	P_{50}	n_{50}
Controls PCO_2 1.06 mm Hg	17.1 ± 0.2 (5)	1.89 ± 0.03 (5)
+ 25 m*M* $NaHCO_3$ PCO_2 5.05 mm Hg	15.4 ± 0.3 (5)[2]	1.82 ± 0.05 (5)
+ Ca $(NO_3)_2$ to equal pCa + pMg in control	16.9 ± 0.5 (6)[3]	1.38 ± 0.05 (6)

[1] Cell respiration method:serum was dialyzed against 0.05 *M* Tris maleate buffered high salinity saline (from Towle and Mangum, 1982). 19.7°C. pH 8.21–8.23. Mean ± S.E. (N).

[2] $P < 0.001$ *vs* control.

[3] $P = 0.05$ *vs* + $NaHCO_3$, 0.75 *vs* control.

requires more CO_2 at high than at low salinity and it can be quantitatively explained by the restoration of total divalent cation activity to levels that existed prior to the addition of CO_2. There is no reason to postulate a binding site linked to the active site for which anions compete. The similar responses in these two species also support the conclusion drawn earlier that the fundamentally different quaternary structures of the molluscan and arthropod Hcs do not mandate different O_2 binding properties (Mangum *et al.*, 1985).

The surprising effect of CO_2 on the Hc of the freshwater crab *Holthuisana* (Greenaway, Bonaventura, and Taylor, 1983; Greenaway, Taylor, and Bonaventura, 1983) may be due to the same phenomenon; this Hc may not be directly sensitive to carbon dioxide. Unlike the response of other crustacean Hcs, the O_2 affinity of this molecule decreases with the addition of molecular CO_2, (9.9 mm Hg), and the effect is slightly greater at pH 7.6 than at 7.22–7.30. Moreover, the levels of divalent cations in the blood of this freshwater crab are presumably relatively low (Greenaway and MacMillen, 1978), even at low levels of CO_2. On the other hand, relatively few of the CO_2 anions would be formed by the addition of 9.9 mm Hg PCO_2 in that pH range (Greenaway, Bonaventura, and Taylor, 1983). Its Hc would have to be especially sensitive to divalent cations.

Typically the slopes of Hill plots of HcO_2 equilibria increase with oxygenation. Since these data are often collected by a tonometric procedure that involves the stepwise addition of unscrubbed room air, we suggest that at least a fraction of the slope change may result from an increase in O_2 affinity with CO_2 rather than oxygenation, especially in species in which cooperativity is not very sensitive to CO_2.

The Cl^- sensitivity of *Penaeus* Hc may be either (1) specific and mimicked exactly by a specific effect of Na^+, at least within the physiological range, or (2) a general effect of ionic strength. The information presently available does not decide the question.

Since CO_2 sensitivity is not related to the sensitivity of inorganic anions (and since the nature of the binding sites is totally unknown), it is not possible at present to predict the effects of CO_2 (or Cl^-) on the O_2 affinity of an unknown Hc. Regrettably, if the knowledge is needed it must be acquired in each case. Since it may be quite large, the effect of Cl^- may be an important factor in understanding how a HcO_2 transport system works. In *Penaeus*, for example, a change from 330 to 560 m*M* Cl^-, well within the physiological range in many crustaceans (though perhaps not in these offshore species), causes a 50% change in P_{50} (pH 7.6). Though more often small, the CO_2 effect may become similarly large at 25°C and physiological pH and Cl^-, the maximum being 46% in the range 0–14.8 mm Hg (*Palaemonetes*). This is considerably greater than the effect of CO_2 on mammalian hemoglobin at the same temperature and at 100 m*M* Cl^- and pH 7.4 (calculated from data shown by Imai, 1982). The effect of CO_2 on Hc cooperativity is probably even more important. At pH 7.6 the maximum is a change of about 100% (*Carcinus*), which could radically alter oxygenation states at both the gill and the tissues. A more quantitative assessment awaits extensive measurements of physiological extremes of PCO_2 made simultaneously with measurements of HcO_2 binding and its other determinants.

ACKNOWLEDGMENT

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EVIDENCE FOR ECDYSTEROID FEEDBACK ON RELEASE OF MOLT-INHIBITING HORMONE FROM CRAB EYESTALK GANGLIA

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ABSTRACT

The content and release of molt-inhibiting hormone activity (MIH) in isolated eyestalk ganglia of crabs (*Cancer antennarius* S.) were measured *in vitro* as a function of exposure *in vivo* to elevated or reduced hemolymph ecdysteroid levels. Ecdysteroid titers of intermolt crabs injected with 20-hydroxyecdysone (two 45 μg injections/24 h) rose 8- to 10-fold; MIH released from subsequently isolated ganglia was significantly less than that released from ganglia of saline-injected controls, while MIH content of ganglia from treated crabs was increased. The hemolymph ecdysteroid level of intermolt crabs was low (6 ng/ml) and was further reduced by 40% 6 days after Y-organectomy. MIH release from ganglia of both Y-organectomized and sham-operated control crabs was high, similar to that of unoperated controls, but MIH content of ganglia from both Y-organectomized and sham groups was significantly reduced relative to controls. The results indicate a negative feedback regulation of MIH release but not synthesis by ecdysteroids and are discussed in relation to the patterns of ecdysteroid titers observed in the normal crustacean molt cycle.

INTRODUCTION

In vertebrates, steroids produced by neuropeptide-regulated glands exert feedback actions on production and/or release of the tropic neurohormones. Feedback effects are generally negative as in adrenal glucocorticoid inhibition of corticotropin-releasing factor and adrenocorticotropin (ACTH) release from the hypothalamus and the pituitary, respectively (see Keller-Wood and Dallman, 1984 for review); feedback of gonadal steroids on gonadotropin release is predominantly negative although positive effects are seen under certain physiological circumstances (Baldwin *et al.*, 1974; Martin *et al.*, 1974; Labrie *et al.*, 1978). Insect neurosecretion of brain prothoracicotropic hormone (PTTH) is apparently subject to inhibition by the ecdysteroid molting hormone, ecdysone, which is produced by the target prothoracic glands (Steel, 1975). In these cases neuropeptide regulation of target cell steroidogenesis is positive, and negative feedback provides a homeostatic mechanism for maintaining circulating steroid hormone levels within a relatively narrow range. The ecdysteroidogenic glands of crustaceans (Y-organs), on the other hand, are subject to negative regulation by the eyestalk neuropeptide molt-inhibiting hormone (MIH; *cf.* Skinner, 1985); the existence of a corresponding feedback arrangement is probable but has not been explored.

The crustacean molt-controlling system consists of eyestalk neurosecretory cells (X-organs, XO) with enlarged axonal endings contained in the neurohemal sinus gland

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Abbreviations. GCS, eyestalk ganglia-conditioned saline; MIH, molt-inhibiting hormone; PTTH, prothoracicotropic hormone; SG, sinus gland; XO, X-organ.

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(SG) and paired peripheral steroidogenic Y-organs which produce ecdysone (Chang *et al.*, 1976; Cooke and Sullivan, 1982; Watson and Spaziani, 1985a). MIH is a heat-stable, trypsin-sensitive peptide (Rao, 1965; Quackenbush and Herrnkind, 1983) which by immunological and functional criteria appears to be related to neurohypophysial peptides of the vasopressin family (Mattson and Spaziani, 1985a). Serotonergic neurons mediate release of MIH (Mattson and Spaziani, 1985b, 1986a) and one of the conditions governing the release of MIH is stress (Mattson and Spaziani, 1985c, 1986a). Recent advances in Y-organ culture methods have allowed demonstration of a direct suppression of Y-organ ecdysteroid production by MIH activity in eyestalk extracts (Watson and Spaziani, 1985b; Mattson and Spaziani, 1985d, Webster, 1986), sinus gland-conditioned saline (Soumoff and O'Connor, 1982; Mattson and Spaziani, 1985b, Webster, 1986), and eyestalk ganglia-conditioned saline (GCS; Mattson and Spaziani, 1985b). *In vitro* methods also show that cyclic AMP mediates this inhibition of steroidogenesis (Mattson and Spaziani, 1985a, e) and that calcium-calmodulin antagonizes the MIH effect by activating Y-organ cAMP phosphodiesterase (Mattson and Spaziani, 1986b). Thus the effects of cAMP on Y-organ steroidogenesis are opposite to those of the tropic hormones on steroidogenic glands of both vertebrates (Schimmer, 1980; Sala *et al.*, 1979) and insects (Smith *et al.*, 1984).

Recent development of a formal MIH bioassay based upon *in vitro* suppression of Y-organ ecdysteroid production (Mattson and Spaziani, 1985d) allowed assessment of neurotransmitter regulation of release of MIH activity from isolated crab eyestalk ganglia (Mattson and Spaziani, 1985b). The present study employs the MIH bioassay and isolated ganglia techniques to assess possible feedback regulation of MIH release by ecdysteroids. Circulating ecdysteroid titers were artificially elevated or suppressed by injection of 20-hydroxyecdysone or Y-organectomy, respectively; eyestalk ganglia were subsequently isolated and ganglionic content and release of MIH was quantified by bioassay.

MATERIALS AND METHODS

Animals and experimental treatment

Female rock crabs *Cancer anternnarius* Stimpson (Marinus Inc., Westchester, California; Pacific Biomarine, Venice, California) were used in all experiments; animals were in intermolt upon sacrifice at the end of experiments (staged by examination of the continuity of endodermis and carapace; *cf.*, Skinner, 1985). Crabs were maintained individually in water table compartments containing constantly-recirculating, charcoal-filtered, reconstituted seawater at 16–17°C. A 12/12 h light/dark photocycle was maintained; crabs were fed fish three times weekly and were allowed to acclimate to their environment for at least one week prior to experimentation to reduce possible stress effects on the XO–Y-organ axis (Mattson and Spaziani, 1985c). Hemolymph samples were withdrawn in 300 μl volumes from the sinus at the base of the fourth walking leg, while treatments were administered by injection (300 μl volumes) through the periarthrodal membrane at the base of the first walking leg. Twenty (20)-hydroxyecdysone was obtained from Sigma Chemical Co. (St. Louis, Missouri) and injected at a dose of 45 μg/300 μl; this concentration (10^{-3} M) was estimated to give an initial hemolymph concentration of 10^{-5} M based upon an average hemolymph volume/crab of 30 ml. Y-organ ablation was carried out on animals chilled for 1 h at 4°C. A 3–5 mm diameter hole was made through the ventral carapace using a portable dental drill with a rough burr bit. Y-organs were ablated by cautery and the carapace opening sealed with paraffin wax. For sham operations, holes were drilled 1 cm distolateral to the Y-organ site and underlying tissue was cauterized. Crabs were examined at sacrifice with the aid of a dissecting microscope to verify Y-organ destruction.

Eyestalk ganglia incubation and extract preparation

At the end of treatment periods eyestalks were extirpated, placed in Pantin's saline (Pantin, 1934), and the entire optic ganglia complexes (including the intact X-organ-sinus gland system) were removed to saline as reported previously (Mattson and Spaziani, 1985b). Ganglia were incubated (1 ganglion complex/100 μl saline) for 2 h at 20°C in an atmosphere of 50% O_2/50% room air with constant rotary shaking at 60 rpm; we previously found that 2-h incubations resulted in release of MIH activity which was intermediate to shorter (30-min) or longer (8-h) incubations and thus allowed for greater sensitivity in detection of changes in release in response to experimental treatments (Mattson and Spaziani, 1985b). After incubations, the ganglia-conditioned saline (GCS) was removed, placed in a boiling water bath for 2 min, and centrifuged at 1000 $\times$ *g* for 10 min. The supernatant volume was adjusted to the preincubation volume with glass distilled water and stored frozen for MIH bioassay. Ganglia extracts were prepared by homogenization in saline (2 ganglia/100 μl) followed by heat treatment in a boiling water bath as previously described (Mattson and Spaziani, 1985d). A dose of two eyestalk equivalents of extract was used for bioassay of MIH; this dose was previously determined to be near the ED_{50} for inhibition of Y-organ steroidogenesis (Mattson and Spaziani, 1985d) and thus allowed for maximal sensitivity of the bioassay to changes in MIH content of ganglia due to experimental treatments.

Bioassay of MIH activity

The MIH bioassay has been previously described (Mattson and Spaziani, 1985b, d). Briefly, activated Y-organs from 48-h de-eyestalked crabs were removed, quartered, and placed in 0.5 ml of fetal bovine serum-supplemented Medium 199. To the incubation medium was then added either 100 μl of saline (for determination of basal ecdysteroid production), eyestalk extract, (2 eyestalk equivalents) or GCS (1 eyestalk equivalent). Incubations were for 24 h, after which incubation medium was removed and stored at 4°C for ecdysteroid RIA, while tissue was processed for protein quantification. The relative ability of GCS or eyestalk extracts (see figure legends) to inhibit Y-organ steroidogenesis was used as a measure of MIH activity (Mattson and Spaziani, 1985b, d). The ecdysteroid contents of GCS and ganglia extracts (at concentrations used for the MIH bioassay) *per se* were below the limit of detection of the ecdysteroid RIA.

Ecdysteroid and protein quantification

Serum and incubation medium were assayed directly for ecdysteroids by RIA as previously described (Mattson and Spaziani, 1985c, d). The RIA utilized ^{3}H-ecdysone (60 Ci/mmol; New England Nuclear, Bedford, MA), ecdysone antiserum (antibody H-21B; Horn *et al.*, 1976) which was a gift from Dr. W. E. Bollenbacher (Dept. of Biology, University of North Carolina, Chapel Hill, NC), and ecdysone standards (Research Plus, Bayonne, NJ). Inter- and intra-assay coefficients of variation were 8% and 4%, respectively. Ecdysone antiserum H-21B has a 10-fold greater affinity for ecdysone than 20-hydroxyecdysone (Horn *et al.*, 1976; Watson and Spaziani, 1985b); as crab hemolymph contains predominantly 20-hydroxyecdysone (Chang *et al.*, 1976) actual ecdysteroid titers for hemolymph in this study are likely an order of magnitude higher than the values presented. The Y-organs of *C. antennarius in vitro* secrete, in addition to ecdysone, an unknown, less polar, ecdysteroid (structural analysis is in progress); this unknown appears in quantities 5-fold greater than ecdysone but has an affinity for antiserum H-21B 100-fold less than ecdysone (Watson and Spaziani, 1985b). Thus, ecdysone levels that we report in the medium of MIH bioassay runs are underrepresented by well under an order of magnitude. Protein was quantified by the Bradford (1976) method. Statistics were done by Students *t*-test and all values are expressed as mean and standard error of the mean (SEM).

RESULTS

Serum ecdysteroid titers of crabs given injections of 45 μg of 20-hydroxyecdysone at 0-h and 18 h later were elevated 3- and 8-fold 18- and 24-h, respectively, after the initial injection (Fig. 1). Y-organectomy reduced hemolymph ecdysteroid levels over a 6-day period to 65% of levels in sham-operated controls (Fig. 2). Eyestalk ganglia removed from crabs with elevated ecdysteroid levels (24 h after initial 20-hydroxyecdysone injection; *cf.* Fig. 1) released significantly less MIH activity during a 2-h incubation in saline than did control ganglia; ganglia-conditioned saline from controls inhibited basal Y-organ steroidogenesis by 42%, while that from crabs with elevated ecdysteroid titers did not affect ecdysteroid production significantly (Fig. 3). Isolated ganglia from Y-organectomized animals with reduced ecdysteroid titers (*cf.*, Fig. 2) released MIH activity at levels similar to ganglia from sham-operated controls or from unoperated controls (Y-organ steroidogenesis was suppressed 40–50%; Fig. 3). Extracts of eyestalk ganglia from the ecdysteroid-treated crabs contained significantly more MIH activity than ganglia extracts from control crabs (Fig. 4); Y-organ steroidogenesis was inhibited 65% and 80% by extracts from control and 20-hydroxyecdysone-injected crabs, respectively. Figure 4 also shows that MIH activity contained in extracts of ganglia from Y-organectomized crabs and from sham operates was significantly less than that in ganglia from unoperated controls (40% suppression of Y-organ ecdyste-

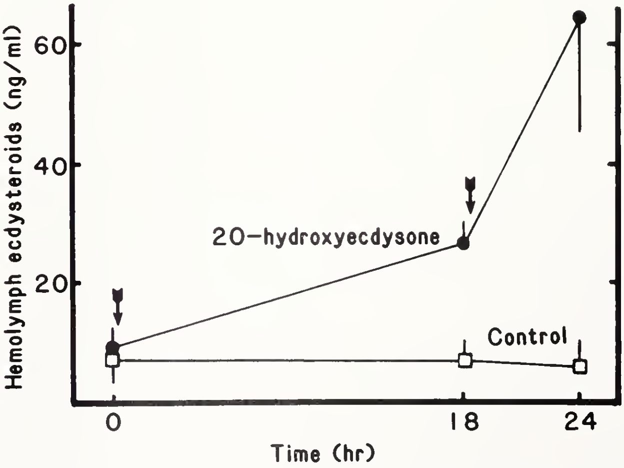

FIGURE 1. Hemolymph ecdysteroid titers after 20-hydroxyecdysone administration. Crabs were injected with saline (control) or 45 μg of 20-hydroxyecdysone (arrows) after serum sampling at the given times. Ecdysteroids were quantified by RIA. Points and lines are the mean and SEM of samples from 4 crabs from 1 of 2 duplicate experiments. Values for 20-hydroxyecdysone-injected crabs > control at 18- and 24-h ($P < 0.01$); 24-h 20-hydroxyecdysone value > 18-h 20-hydroxyecdysone value ($P < 0.05$).

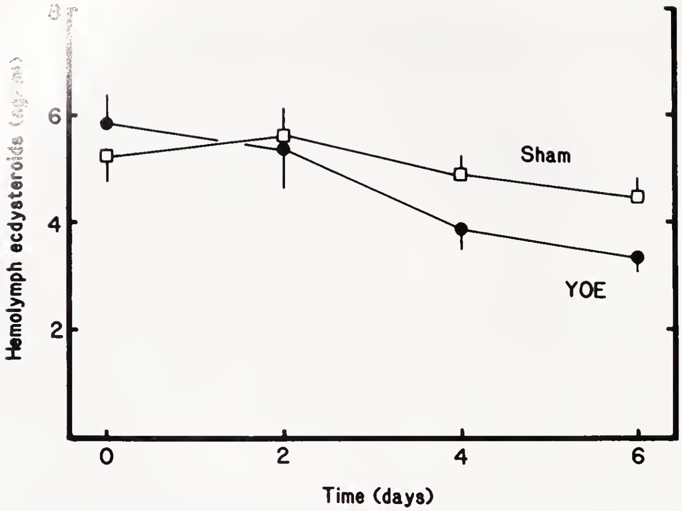

FIGURE 2. Effects of Y-organ removal on hemolymph ecdysteroid levels. Y-organectomy or sham operations were performed after serum sampling at time 0; hemolymph ecdysteroid levels were monitored at the given times thereafter. Points and lines are the mean and SEM from 4 to 5 crabs. Values for Y-organectomized crabs < corresponding sham values at days 4–6 ($P < 0.05$); combined values for sham days 4 and 6 < sham day 2 value ($P < 0.05$).

roidogenesis for ganglia extracts from Y-organectomized and sham groups; 65% suppression for ganglia extracts from unoperated controls).

DISCUSSION

We found that injection of 20-hydroxyecdysone increased circulating ecdysteroid titers. The same was found by Adelung (1967) after injecting intermolt crabs with a single dose of ecdysone, followed by extraction of whole animals at intervals and measuring ecdysteroid by the Calliphora assay. He also observed that the ecdysteroid level fell rapidly within the first 4 hours after injection, to 10% of the administered dose, and then, surprisingly, rose again over the subsequent 18 hours to the 50% level before finally declining. These events were interpreted to result first from a rapid clearing of injected ecdysone and then endogenous secretion of ecdysone by the Y-organs, directly stimulated by the injected hormone through a positive feedback mechanism. That the source of the secondary rise in ecdysteroid was endogenous was unequivocally demonstrated: an injection of ^{3}H-ecdysone was cleared rapidly over 4 hours as before but levels of the tracer continued downward over the subsequent time period (Adelung, 1967). In the present study, we show that elevated ecdysteroid levels in crab hemolymph inhibit release of MIH activity from subsequently isolated eyestalk ganglia (Fig. 3). It appears that the better interpretation of Adelung's results, based on present knowledge, is that the secondary rise in ecdysteroids he observed was due to transient inhibition of MIH release induced by the ecdysteroid injection.

A methodology similar to that employed in the present study was used to demonstrate feedback inhibition of ACTH release from rat pituicytes *in vitro* (Mulder and Smelik, 1977); ACTH release from pituicytes of corticosterone-treated rats was measured with a bioassay based on stimulation of steroidogenesis by cultured adrenal cells. In that experiment pituitary cells exhibited the feedback effects for several hours after

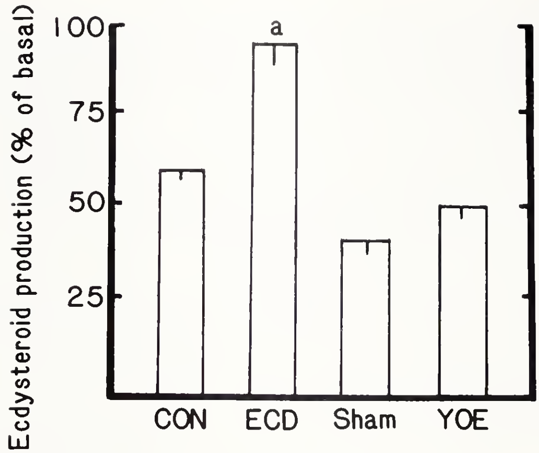

FIGURE 3. Effects of elevated and reduced hemolymph ecdysteroid titers on release of MIH from isolated eyestalk ganglia. Ganglia (removed from the crabs used in the Figs. 1 and 2 experiments) were incubated 2 h in saline, and MIH activity in the conditioned saline was assessed by ability to inhibit Y-organ steroidogenesis. Saline was conditioned with ganglia from untreated control crabs (CON), from crabs with elevated ecdysteroid titers (ECD), from sham-operates (Sham), or from Y-organectomized crabs (YOE). Bars and enclosed lines represent the mean and SEM of 8–10 incubations. Basal ecdysteroid production by Y-organ quarters (in unconditioned saline) averaged 155 ± 12 pg/μg protein/24 h. a, $P < 0.01$–0.001 *vs* CON. Sham *vs* CON, $P < 0.01$.

isolation, indicating a relatively long-lasting influence of corticosteroid exposure *in vivo.* Similarly, eyestalk ganglia isolated from ecdysteroid-treated crabs apparently remained suppressed during 2-h incubations as MIH activity was not detectable in

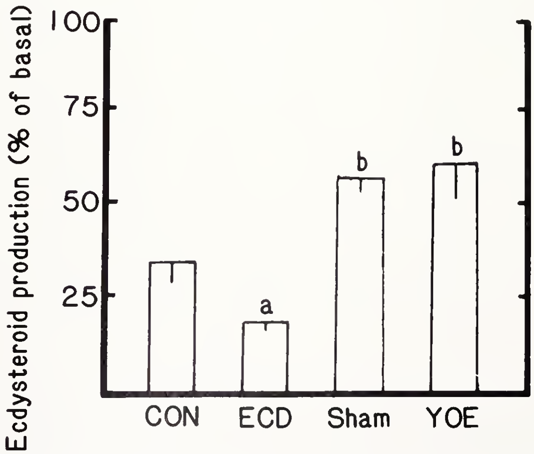

FIGURE 4. Effects of elevated and reduced hemolymph ecdysteroid levels on MIH content of eyestalk ganglia. Y-organ quarters were incubated with extracts of ganglia (2 eyestalk equivalent) from untreated control (CON) crabs, from crabs with elevated ecdysteroid levels (ECD), from sham operates (Sham), or from Y-organectomized crabs (YOE); effects on ecdysteroidogenesis were measured. Bars and enclosed lines represent the mean and SEM of 8–10 incubations. Basal ecdysteroid production by gland quarters averaged 83 ± 12 pg ecdysteroids/μg protein/24 h. All treatments suppressed ecdysteroid production ($P < 0.01$–0.001). a. $P < 0.01$ *vs* CON b. $P < 0.02$–0.01 *vs* CON; $P < 0.001$ *vs.* ECD.

the conditioned saline (Fig. 3). In a previous study we found that as little as 0.01 eyestalk equivalent of MIH activity released from control ganglia in 2-h incubations elicited detectable suppression of Y-organ steroidogenesis; MIH release from feedback-inhibited ganglia was therefore likely less than 1% of levels released from control ganglia. In addition, ganglia from crabs with elevated ecdysteroids contain significantly more MIH activity than ganglia from control intermolt crabs (Fig. 4). The combined results suggest a primary negative feedback effect on MIH release relative to synthesis. Comparable effects were seen in adrenal corticoid feedback on ACTH release; Jones *et al.* (1977) found that corticosteroids inhibited release of ACTH from cultured pituitary cells without affecting its synthesis.

Y-organectomized crabs showed only a 40% reduction in circulating ecdysteroid levels (Fig. 2). Other studies in which Y-organs were ablated and serum ecdysteroid levels were monitored yielded similar results (see Skinner, 1985 for review); one study suggested that ecdysteroids released by ovaries may account for the maintenance of low levels of ecdysteroids in the absence of Y-organs (Lachaise and Hoffman, 1977). While the reduction of ecdysteroid levels caused by Y-organectomy did not affect MIH release (Fig. 3), MIH content of ganglia from Y-organectomized and from sham-operates was reduced (Fig. 4). In light of previous studies indicating that stressors reduce ecdysteroid titers by promoting MIH release (Mattson and Spaziani, 1985c, 1986a) and prevent molting (Aiken, 1969), we propose that stress due to surgery may have caused a large release of MIH prior to post-surgery day 6, resulting in depletion of ganglionic MIH content and low amounts of MIH available for further release.

Serum ecdysteroid levels during crab molting cycles have been determined in several species (Adelung, 1967, 1969; Chang, *et al.*, 1976; Hopkins, 1983; Soumoff and Skinner, 1983; Skinner, 1985) and follow a consistent pattern. Associated with the transition from intermolt to premolt is one or more relatively small, transient rises in hemolymph ecdysteroid titers (3- to 10-fold over intermolt levels). There follows a dramatic rise (100- to 300-fold) that precedes, and presumably initiates, ecdysis. Titers then fall sharply just prior to ecdysis and return shortly thereafter to or below intermolt levels. Results of the present study and of previous work in this and other laboratories suggest interactions of the XO-SG-Y-organ neuroendocrine system that may account for the observed changes in hemolymph titers. The small, pre-ecdysial rises may result from preliminary reduction in MIH secretion due to internal or external environmental cues (*e.g.*, changes in photoperiod and/or temperature, Bliss and Boyer, 1964, Aiken, 1969; intrinsic neuronal oscillators that are species specific, Arechiga *et al.*, 1985; reductions in stressful inputs, Mattson and Spaziani, 1985c, 1986a). Once initiated, the small ecdysteroid peaks may create changes in activity of eyestalk neurosecretory cells or their inputs resulting in feedback inhibition of MIH release (*cf.* Fig. 5). The large secondary rise in ecdysteroids is consequently permitted, causally linked to accentuated feedback inhibition of MIH release (but not synthesis). The magnitude of this rise in ecdysteroids may be explained in part by our recent findings (Mattson and Spaziani, 1986b) that calcium antagonizes MIH suppression of Y-organ activity. It is well documented (*cf.* Greenaway, 1985) that hemolymph calcium titers rise sharply prior to the molt and fall agin, more or less coincident with the changes in ecdysteroids. Thus, both the pre-ecdysial rise and fall in ecdysteroids also may be calcium-linked. In any case, with ecdysteroid levels finally depressed, feedback inhibition on neurosecretory cells would be relieved and MIH release reinstated to dominate the postmolt and intermolt hormonal environments. However, while this scenario may be plausible for crustaceans, it is not apparently consistent with all events in insects, which exhibit the same general pattern of cyclic changes in ecdysteroid levels (*cf.*, Bollenbacher *et al.*, 1975). Steel (1975) provided evidence of negative feedback effects of ecdysteroids

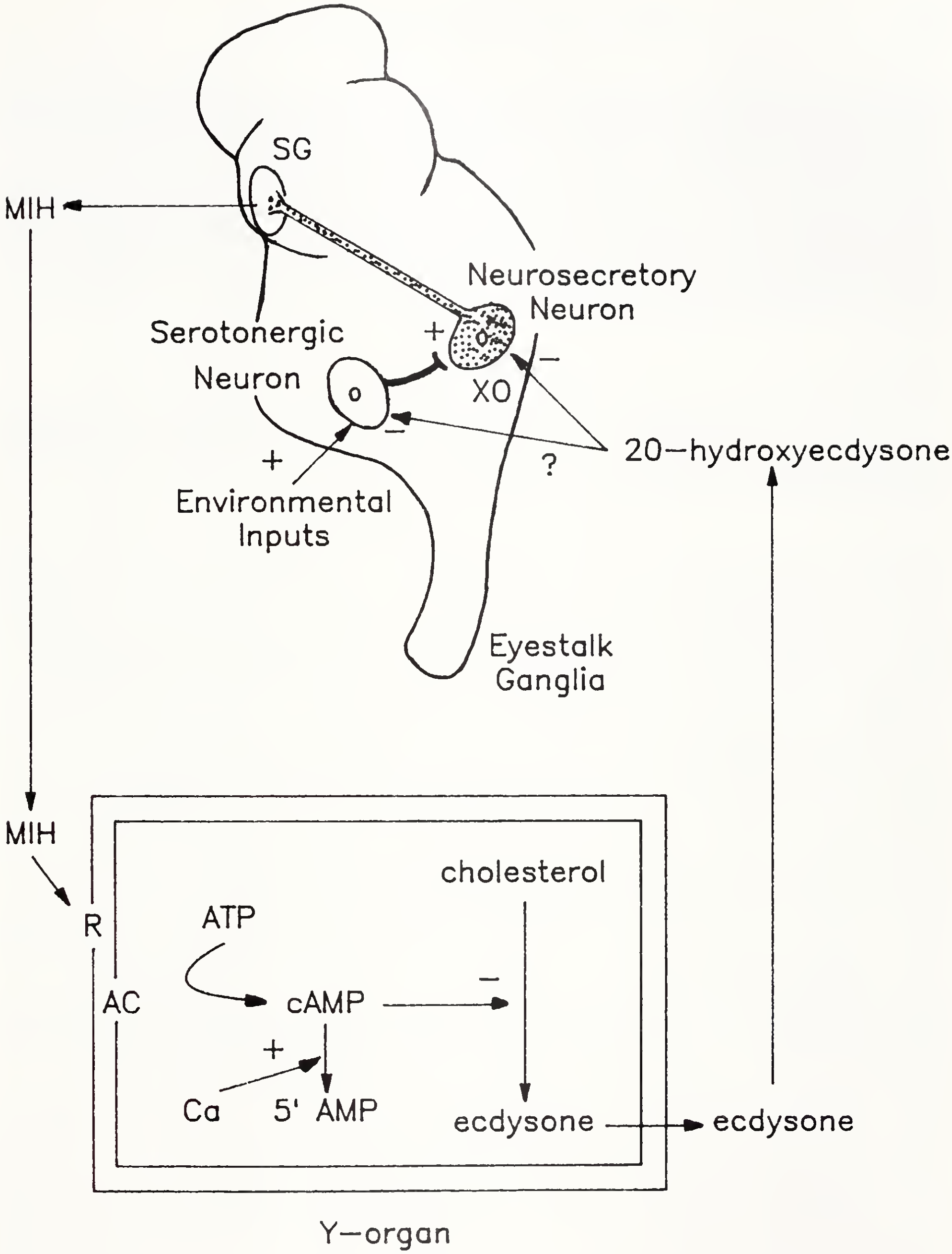
SG
MIH
Neurosecretory Neuron
Serotonergic Neuron
+
XO
–
20–hydroxyecdysone
?
+
Environmental Inputs
Eyestalk Ganglia
MIH
R
AC
ATP
cAMP
–
cholesterol
+
Ca
5' AMP
ecdysone
ecdysone
Y–organ

FIGURE 5. Model of neuroendocrine regulatory interactions of the X-organ-sinus gland-Y-organ system. Environmental inputs including stress activate serotonergic eyestalk neurons which stimulate MIH-containing neurosecretory cells of the X-organ (XO)-sinus gland (SG) complex to release MIH. MIH in hemolymph binds to putative Y-organ cell surface receptors (R) resulting in activation of adenylate cyclase (AC) and generation of cAMP. cAMP suppresses production of ecdysone from cholesterol, an effect antagonized by calcium (Ca) which activates a calcium-calmodulin-sensitive cAMP-phosphodiesterase. Thus with continued release of MIH, ecdysone titers remain low and the intermolt state is maintained. A reduction of MIH release (due to a transient increase in hemolymph ecdysteroid levels or to reduced peripheral neural input) releases Y-organs from inhibition (decreased Y-organ cAMP levels) and ecdysone production is increased. Ecdysone is converted in peripheral tissues to 20-hydroxyecdysone. The latter eventually exerts feedback inhibition on release of MIH from XOSG cells, permitting the large rise in hemolymph ecdysteroid titers prior to the molt. Y-organ activity subsequently declines (cause unknown), ecdysteroid titers fall, and MIH is again released reinstating the intermolt stage. See text for further discussion and details.

on release of the insect tropin, PTTH. The problem of consistency in model arises from the fact that the insect tropin stimulates ecdysteroid secretion whereas the crustacean tropin is inhibitory. Thus, the initial rises in ecdysteroids in insects, and the subsequent large increase, would be expected to result from a stimulation, not suppression, of PTTH release. In further contrast with crustaceans, the subsequent pre-ecdysial fall in ecdysteroids is more satisfactorily explained in insects by the feedback inhibition hypothesis. Clearly, resolution of these questions must await the development of a sensitive means for measuring arthropod tropic hormone levels in hemolymph as a function of the molting cycle.

ACKNOWLEDGMENTS

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ADAPTATIONS TO SULFIDE BY HYDROTHERMAL VENT ANIMALS: SITES AND MECHANISMS OF DETOXIFICATION AND METABOLISM

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ABSTRACT

The detoxification and metabolism of sulfide were studied in three symbiont-containing invertebrates from the deep-sea hydrothermal vents: the tube worm, *Riftia pachyptila;* the clam, *Calyptogena magnifica;* and the mussel, *Bathymodiolus thermophilus.* Sulfide oxidizing activities, due to specific sulfide oxidase enzymes, were found in all tissues, with the greatest activities occurring in the symbiont-containing tissues: the trophosome of the tube worm and the gills of the bivalves. Sulfide oxidase activity was correlated with the bacterial content of the tissues. The sulfide oxidases in the outer cell layer(s) of symbiont-free tissues, *e.g.*, body wall muscle of *Riftia* and foot and mantle of the bivalves, may detoxify sulfide as soon as it enters the body. Sulfide entering the blood in *Riftia* and *Calyptogena* may be bound by sulfide-binding factors that transport sulfide to the symbionts and protect against sulfide inhibition of aerobic respiration [via effects on the cytochrome-c oxidase (CytOx) system]. Sulfide strongly inhibited the CytOx systems of these animals, but this inhibition was offset by the addition to the CytOx assay mixture of blood of *Riftia* or *Calyptogena.* Reduced sulfur compounds, sulfide, sulfite, and thiosulfate, were effective in stimulating ATP synthesis in homogenates of symbiont-containing tissues. The most effective reduced sulfur compound varied among the three symbioses.

INTRODUCTION

Symbioses between marine invertebrates and sulfur-metabolizing bacteria have been discovered in a variety of habitats that are rich in hydrogen sulfide, including the deep-sea hydrothermal vents and many shallow habitats rich in decomposing organic materials (Cavanaugh *et al.*, 1981; Felbeck, 1981, 1983; Felbeck *et al.*, 1981; Felbeck and Somero, 1982; Cavanaough, 1983; Fisher and Hand, 1984; Powell and Somero, 1985). These discoveries have raised two major questions concerning the metabolism of sulfide and other reduced sulfur compounds in these organisms. First, how are aerobic metabolic processes maintained in the face of sulfide concentrations that are high enough to poison completely the cytochrome-c oxidase (CytOx) systems of organisms from sulfide-free environments? The discovery that animals from these sulfide-rich habitats contain CytOx activities comparable to those found in related species from low-sulfide environments (Hand and Somero, 1983) suggests that sulfide detoxification is an important adaptation in these species (*cf.*, Powell and Somero, 1983, 1985).

A second question about the roles of reduced sulfur compounds in these symbioses concerns where and how these energy-rich molecules are metabolized to allow efficient exploitation of their bond energy. In the gutless protobranch clam, *Solemya reidi,* which occurs in shallow water sulfide-rich habitats (pulpmill effluent areas and sewage

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outfall zones), the initial metabolism of sulfide in the symbiont-containing gills appears to occur not in the bacterial symbionts, but rather in specific organelles of animal origin we have termed sulfide oxidizing bodies (Powell and Somero, 1985). It is not known whether the initial step(s) in sulfide oxidation in other symbioses between marine invertebrates and sulfur bacteria occur in these organelles or, instead, within the bacterial endosymbionts. The type of reduced sulfur compound supplied to the bacteria by the host may influence the types of metabolic pathways present in the symbiont and the net ATP yield available from oxidation of sulfur compounds.

During the Galapagos '85 Expedition (March 1985) to the hydrothermal vent site located at the Galapagos Rift (0°N, 86°W) at a depth of approximately 2500 m, we examined several aspects of the sulfide metabolizing and detoxifying mechanisms of three symbiont-containing species that contribute importantly to the vent biomass: the vestimentiferan tube worm, *Riftia pachyptila* (Phylum Pogonophora); the vesicomyid clam, *Calyptogena magnifica;* and the mussel, *Bathymodiolus thermophilus.* We sought to determine for each species the sulfide oxidizing potentials of symbiont-containing and symbiont-free tissues; whether this oxidation was due to specific enzymes (sulfide oxidases; Powell and Somero, 1985); what the aerobic potentials of the species' tissues were and how aerobic respiration was protected from sulfide; and how effective sulfide and other reduced sulfur compounds (sulfite and thiosulfate) were in driving ATP synthesis in the symbiont-containing tissues of the species. Our results show that the species differ in their capacities for sulfide oxidation, in their capacities for aerobic respiration, in the mechanisms used for protecting aerobic respiration from poisoning by sulfide, and in the types of reduced sulfur compounds that are most effective in stimulating ATP generation. Thus, while these three symbioses share major features in common, they also differ strikingly in the mechanisms used to detoxify and exploit sulfide.

Materials and Methods

Experimental animals

Organisms were collected during the 1985 expedition to the Galapagos Rift "Rose Garden" hydrothermal vent site (Hessler and Smithey, 1983). The three species studied, *Riftia pachyptila,* the large vestimentiferan tube worm (Jones, 1981); *Calyptogena magnifica,* a vesicomyid clam (Boss and Turner, 1980); and *Bathymodiolus thermophilus,* the vent mussel (Kenk and Wilson, 1985) are endemic to the vents and are the dominant contributors to the faunal biomass at the Rose Garden site. Specimens were retrieved using the submersible DSRV Alvin operated by the Woods Hole Oceanographic Institution. Freshly collected animals were placed in a thermally insulated container held in the front basket of the submersible and brought to the surface within 2–3 h of collection. Animals were not held at *in situ* pressures (approx. 250 atm) during ascent, but the temperature of the container stayed below approximately 15°C, a temperature readily tolerated by the animals, which experience temperatures of between 2 and approximately 20°C *in situ* (Hessler and Smithey, 1983). The animals were immediately placed in 4°C seawater upon retrieval and held in a refrigerated (approx. 4°C) van on board the RV Melville.

For most experiments tissue samples were dissected from the freshly retrieved samples within 1–3 h of their arrival at the surface. For some studies tissues were removed from animals that had been held at *in situ* pressures for one to several days after capture.

Preparation of solutions of sulfide

At pH values near neutrality, sulfide exists primarily as HS^- and H_2S; only small amounts of $S^=$ are present. We use the term "sulfide" to refer to all sulfide species in solution.

All sulfide solutions were freshly prepared immediately before an experiment, and used within 1–3 h of preparation. To prepare a stock solution, a freshly washed crystal of sodium sulfide was weighed using the motion-compensated shipboard balance system designed by Childress and Mickel (1980), and dissolved immediately in distilled water.

Enzyme assays

Sulfide oxidation was assayed photometrically (at 578 nm) by following the reduction by sulfide of the artificial electron acceptor benzyl viologen (BV) using the assay system of Powell and Somero (1985). The other enzymes studied, cytochrome-c oxidase (CytOx), citrate synthase (CS), and malate dehydrogenase (MDH), were assayed according to the methods of Hand and Somero (1983). The standard assay pH for CytOx was 6.0; different pH values were used under certain circumstances, as indicated in the figures. Partial purification of CytOx was performed as described in Powell and Somero (1983). All enzymatic activities were measured at 20°C. Activities are expressed as international units (umoles substrate converted to product per minute) per g fresh weight (gFW) of tissue.

Microscopy

Sectioning of tissues and staining for sulfide oxidizing activity were performed as described in Powell and Somero (1985), except that the staining buffer for trophosome tissue was 80% artificial seawater (ASW) buffered with 0.2 *M* glycine, pH 9.0, containing 5 m*M* sulfide and 2 m*M* benzyl viologen. One hundred percent ASW was made of NaCl (0.47 *M*), KCl (10 m*M*), $CaCl_2$ (10 m*M*), and $MgCl_2$ (50 m*M*).

Heating of enzyme preparations

The thermal stabilities of the sulfide oxidizing activities were studied by heating unfractionated homogenates of the tissues (trophosome and body wall of *Riftia,* and gills of *Calyptogena* and *Bathymodiolus*) at 90°C for 10 min, and then assaying for sulfide oxidizing activity.

Determining bacterial counts in homogenates

Connective tissue and blood vessels were removed as completely as possible from the tissue samples before use. Tissues were homogenized gently with a Dounce homogenizer, employing three strokes by hand with a loose pestle, and three strokes by hand with a tight pestle. The homogenization buffer was ASW buffered with 20 m*M* Hepes, pH 7.4. *Riftia pachyptila* trophosome samples were counted immediately using a hemocytometer; the large size of the bacteria (2–6 μm) permitted this method of counting. To verify that this cell counting method was accurate, samples of trophosome homogenates were fixed in 5% formalin in ASW Hepes. These samples were stained with D.A.P.I. and counted by epifluorescence microscopy upon return to Scripps. Epifluorescence counts of these trophosome samples confirmed that the earlier hemocytometer counts were accurate.

Size of bacteria and bacterial composition of tissues

Gill tissues were fixed and embedded for microscopy as described by Powell and Somero (1985). One to five μm thick sections were cut, and the size of the bacteria was measured by light microscopy. Tissue composition (% bacteria) was estimated according to the following formula: (% bacteria = # of bacteria per gram fresh weight (gFW) × mass of bacteria × 100). Mass of bacteria = density of bacteria × volume of bacteria. Density of bacteria was assumed to be 1.0 g/c.c volume of bacteria = $\frac{4}{3} \times \pi \times (\text{radius of bacteria})^3$, (all bacteria were approximately spherical).

ATP synthesis studies

Tissues were homogenized in either 20 m*M* potassium phosphate buffer, pH 7.4 (hypoosmotic buffer for lysis of bacteria), or ASW/Hepes, pH 7.4 (isosmotic buffer to prevent bacterial lysis). Samples were examined by phase contrast microscopy for intactness of the bacteria. Reactions were performed at 20°C. The reaction mixture (total volume 2.2 ml), contained 1 m*M* of the reduced sulfur compound serving as the substrate (experimental) or no substrate (control), and homogenate volumes containing 0.022–0.20 gFW tissue. The reaction buffer was 100 m*M* potassium phosphate buffer, pH 7.4 (lysed samples), or ASW/Hepes, pH 7.4 (intact samples). Homogenate and buffer were mixed and allowed to equilibrate for 2 min at 20°C. For the 0 min time point, 0.4 ml of the reaction mixture was removed and quenched with an equal volume of 1.5 *M* $HClO_4$ with rapid mixing; the substrate (sulfate, sulfite, or thiosulfate) was added after quenching. Substrate was then added to the rest of the mixture, and 0.4 ml of the complete reaction mixture was added to each of four test tubes, which were held at 20°C for different lengths of time. At the times indicated in Figure 6, the 0.4 ml aliquots were quenched with an equal volume of 1.5 *M* $HClO_4$. The quenched samples were held for 10 min at room temperature to allow complete denaturation of the proteins in the sample. The precipitated samples were next placed on ice for 10 min, and then centrifuged for 5 min at 10,000 × *g*. The supernatant was neutralized with cold 2 *M* KOH/1 *M* triethanolamine, left on ice for at least 10 min or frozen for up to 6 hours, and centrifuged for 5 min at 10,000 × *g*. The supernatant was then assayed immediately for ATP concentration with firefly luciferase as described (Karl and Holm-Hansen, 1978).

RESULTS

Sulfide oxidation activities

Using the benzyl viologen assay for measuring photometrically the oxidation of sulfide, we detected sulfide oxidizing activities in all of the tissues that were examined (Table I). Activities were highest in the symbiont-containing tissues: the gills of the bivalves and the trophosome of *Riftia.* The latter tissue contained by far the highest levels of sulfide oxidizing activity, and this activity varied along the length of the trophosome in a consistent manner, increasing from top to bottom. The gills of *Bathymodiolus* contained the lowest levels of sulfide oxidizing activity of any symbiont-containing tissue.

The sulfide oxidizing activities listed in Table I represent averages for the entire tissue sample used in the homogenate, and do not indicate whether activity was uniformly distributed throughout a tissue or localized in a precise region. To investigate the localization of sulfide oxidizing activity we used the benzyl viologen histochemical staining procedure.

TABLE I

Sulfide oxidizing activities of tissues of vent invertebrates

Sample	Units × gFW^{-1}
Riftia pachyptila	
trophosome	
top	22.04 ± 7.14 (12)
middle	31.66 ± 8.80 (8)
bottom	40.31 ± 12.30 (13)
plume	0.24 ± 0.09 (8)
body wall	0.44 ± 0.20 (10)
Bathymodiolus thermophilus	
gill	0.98 ± 0.33 (21)
foot	0.18 ± 0.05 (7)
mantle	0.41 ± 0.11 (7)
Calyptogena magnifica	
gill	6.06 ± 2.09 (22)
foot	0.58 ± 0.14 (8)
mantle	0.95 ± 0.41 (5)

Sulfide oxidizing activities of supernatants were measured using the benzyl viologen photometric assay system, and are expressed as units of activity per g fresh weight of tissue. Values expressed as mean ± standard deviation (number of individuals).

Figure 1 illustrates the staining patterns observed in trophosome and body wall of *Riftia* and in foot and mantle of *Calyptogena*. Foot and mantle of *Bathymodiolus* exhibited the same staining patterns found for *Calyptogena* (data not shown). In trophosome all staining was associated with the bacterial endosymbionts. Bacteria were always present in interconnected sausage-shaped lobes. Layers of green structure always surrounded the lobes of bacteria, but the thickness of the green layers was variable; approximately average thickness is shown here. These green layers were responsible for the striking intense green color of trophosome tissue. The green structures were present within animal cells. The bacteria appeared to be endosymbionts, *i.e.*, they were within animal cells. In the body wall muscle of *Riftia* and the foot and mantle tissues of the bivalves—tissues which lack symbionts—the sulfide oxidizing activity was located in the outer cell layers of the tissue, *i.e.*, in the cells in direct contact with the sulfide-laden seawater. So localized, the sulfide oxidizing activity may serve as a "peripheral defense" by rapidly oxidizing any sulfide that penetrates the outer body surface.

Because of the very small size of the bacterial symbionts of the bivalves, histochemical staining of gill tissue could not reveal the precise localization of the sulfide oxidizing activities. Sulfide oxidizing activity did co-purify with the bacteria in tissue fractionation studies employing differential centrifugation (authors' unpub. obs.). However, the bacterial pellets also contained fragments of mitochondria, so we could not determine unambiguously where the sulfide oxidizing activities were localized (see Discussion).

The oxidation of hydrogen sulfide can be catalyzed by a variety of factors besides specific sulfide oxidase (SOx) enzymes like those found in the clam, *Solemya reidi* (Powell and Somero, 1985). Three types of evidence suggest that the sulfide oxidizing activities found in the symbiont-containing and symbiont-free tissues of these animals were specific sulfide oxidase enzymes. First, the sulfide oxidizing activities were significantly higher than those characteristic of sulfide oxidation due to non-specific factors

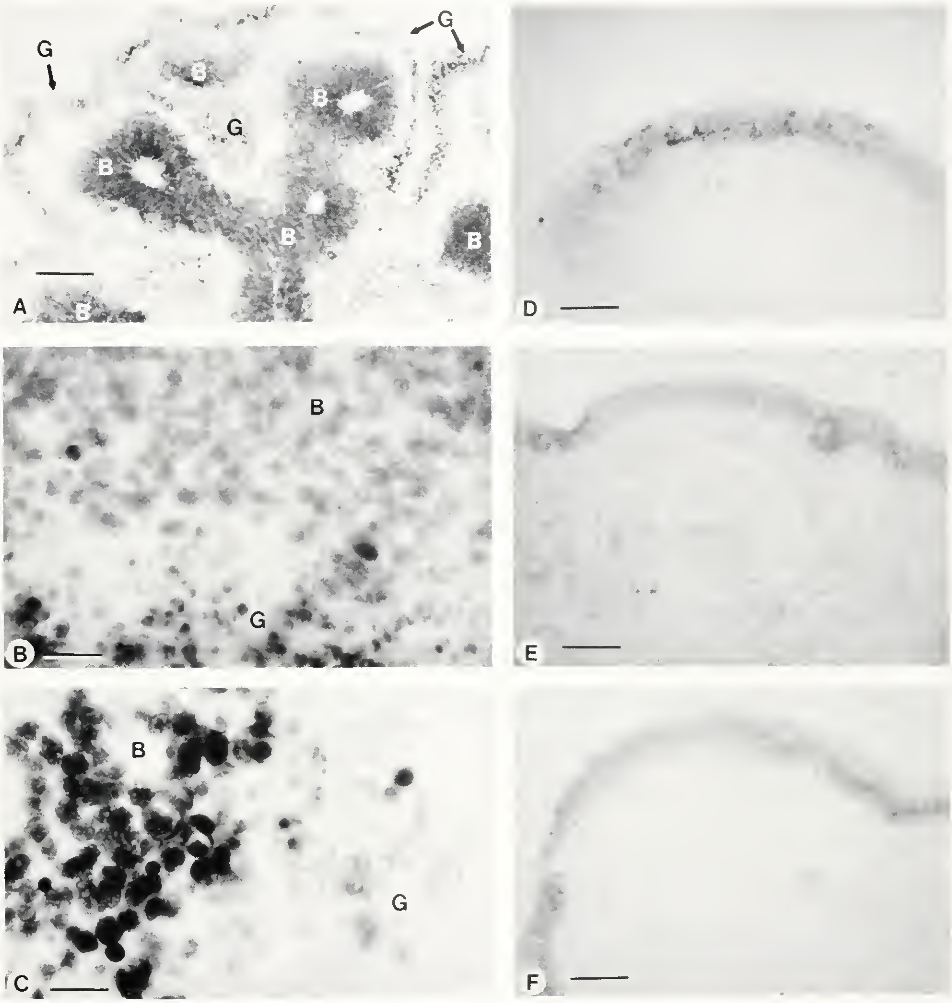

FIGURE 1. Light micrographs of transverse sections through tissues of vent animals. Sections were unstained (A, B), or stained for sulfide oxidation activity (C–F). (A) Low magnification view of unstained *Riftia pachyptila* trophosome tissue. B indicates bacteria and G indicates green structures. Bacteria were filled with translucent sulfur globules (see Fig. 1B), and appear dark at low magnification. Scale bar = 100 μm. (B) High magnification view of unstained trophosome tissue. Bacteria were filled with translucent sulfur globules. Scale bar = 10 μm. (C) High magnification view of trophosome tissue stained for sulfide oxidase activity. Bacteria were stained dark purple (positive for sulfide oxidase activity). Green structures were unstained. Some sulfur globules are still visible. Scale bar = 10 μm. (D) *Riftia pachyptila* body wall tissue stained for sulfide oxidase activity. The surface layer of cells stained positively. Scale bar = 40 μm. (E) *Calyptogena magnifica* mantle tissue stained for sulfide oxidase activity. The surface layer of cells stained positively. Scale bar = 40 μm. (F) *Calyptogena magnifica* foot tissue stained for sulfide oxidase activity. The surface layer of cells stained positively. Controls (benzyl viologen reagent but no sulfide) did not exhibit any staining. Scale bar = 40 μm. Abbreviations: B = bacteria, G = green structures.

(*cf.*, Powell and Somero, 1985). Second, we found that the activities were heat labile, with rapid loss of sulfide oxidizing activity occurring during incubation at 90°C (see Materials and Methods; data not shown). Third, the activities displayed Michaelis-

Menten kinetics with respect to sulfide (*cf.*, Powell and Somero, 1985; Powell and Somero, in prep.). We conclude, therefore, that specific sulfide oxidase enzymes were present in both the symbiont-containing and the symbiont-free tissues of these animals. Characterization of these enzymes will be reported in a subsequent paper (Powell and Somero, in prep.).

Activities of enzymes of energy metabolism

We surveyed the tissues of the three species for the activities of enzymes that serve to diagnose the types of pathways of energy metabolism operating to generate ATP. CytOx activity is an indicator of the capacity for electron transport chain activity, *i.e.*, for aerobic respiration; citrate synthase (CS) is an indicator of citric acid (Krebs) cycle activity. Malate dehydrogenase (MDH) is involved in several metabolic functions [citric acid cycle activity; the transfer of reducing equivalents across the mitochondrial membrane; and in the anaerobic pathway involving the channeling of phosphoenolpyruvate to succinate, via oxaloacetate, malate, and fumarate (Hochachka and Somero, 1984)].

CS activities were uniformly higher in tissues of *Riftia* than in the two bivalves, indicating a higher capacity for citric acid cycle function in the tube worm than in the clam or mussel. CytOx activities also were much higher on the average in *Riftia* than in the bivalves, suggesting that the tube worm has a more aerobic poise to its energy metabolism than the clam or mussel. MDH activities varied by less than an order of magnitude among all of the tissues examined (Table II). The finding that a much higher ratio of MDH to CS (and CytOx) characterized the tissues of the two bivalves relative to *Riftia*, suggests that the MDH in the bivalve tissues may be principally important in the context of anaerobic metabolism. In trophosome of *Riftia* there was no systematic variation in MDH or CS activities with position, unlike the pattern noted for SOx activity (Table I).

Protection of CytOx activity by blood

In addition to a detoxification strategy that relies on rapid oxidation of sulfide when it first enters the body, protection of aerobic respiration also may depend on mechanisms for detoxifying sulfide that passes through the body surface and enters the circulation. We earlier showed (Powell and Somero, 1983) that the blood of *Riftia* was capable of offsetting the inhibition of CytOx activity caused by sulfide. To investigate protection by blood in more detail we further characterized the protective effects of *Riftia* blood and also studied blood of the clam, *Calyptogena*.

The importance of mechanisms for protecting CytOx activity from inhibition by sulfide is emphasized by the data in Figure 2, which show that the CytOx systems of *Riftia* and *Bathymodiolus* are highly sensitive to sulfide, as is the CytOx of *Calyptogena* (see Fig. 3). The CytOx systems of *Riftia* and *Bathymodiolus* show identical sensitivities to sulfide (pH 6.0 data), with virtually complete inhibition occurring by sulfide concentrations of 20–50 μM. The sensitivity of CytOx to sulfide is strongly dependent on pH, and the stronger effects noted at pH 6.0 suggest that H_2S is the inhibitory form of sulfide.

Bloods of *Riftia* and *Calyptogena* were able to counteract the inhibition of CytOx activity by sulfide (Fig. 3). The addition of small quantities of freshly collected blood to the CytOx assay system reduced the inhibitory effects of sulfide on the activities of the enzymes of *Riftia* and *Calyptogena*. The protective effects of blood of *Riftia* appear from the data in Figure 3 to be greater than those of *Calyptogena* blood. However,

TABLE II

Enzymatic activities in tissues of three vent invertebrates

Sample	Units × gFW^{-1}			
	MDH	CS	CytOx	SOx
Riftia pachyptila				
plume	28.1	4.29	8.32	nd
vestimentum	40.4	2.53	2.54	nd
body wall	63.6	10.00	1.27	nd
trophosome				
top-1	29.7	1.53	nd	31.7
top-2	30.2	0.65	nd	33.8
top-3	21.3	1.88	nd	19.7
bottom-1	41.8	1.23	nd	49.7
bottom-2	23.9	0.71	nd	56.4
bottom-3	24.8	1.12	nd	56.1
Bathymodiolus thermophilus				
gill-1	41.8	0.02	0.89	nd
gill-2	38.0	0.02	1.54	nd
adductor-1	54.3	0.02	0.41	nd
adductor-2	39.0	0.03	0.27	nd
foot-1	35.7	0.02	0.12	nd
foot-2	33.4	0.02	0.22	nd
mantle-1	27.8	0.01	0.14	nd
mantle-2	25.8	0.01	0.08	nd
Calyptogena magnifica				
gill-1	39.2	0	0.41	nd
gill-2	37.1	nd	nd	5.15
gill-3	42.5	nd	nd	8.94
gill-4	32.0	nd	nd	8.67
gill-5	34.8	nd	nd	6.63
gill-6	34.1	nd	nd	5.29
adductor	116.9	0.41	0.11	nd
foot	25.1	0.18	0	nd
mantle	23.2	0.12	0	nd

"nd" means not determined. "0" refers to no detectible activity. Each numbered tissue refers to a tissue sample from a different individual.

the protocols used for obtaining the CytOx systems of the two species (plume of *Riftia* and heart of *Calyptogena*) differed, and the sensitivity of CytOx to sulfide is strongly dependent on the extent to which the enzyme is purified (Powell and Somero, 1983). The purification scheme used for the *Riftia* enzyme inactivated the enzyme from *Calyptogena,* so we were forced to use a crude homogenate to assay the CytOx activity of clam heart. Therefore, the apparent difference in protective ability between the two bloods may, in fact, be entirely a reflection of the state of purity of the CytOx preparations. Therefore, the data in Figure 3 should be taken only as evidence that both bloods can protect CytOx, and not as quantitative estimates of this protective capacity.

The abilities of blood (*Riftia*) to protect the CytOx system from poisoning by sulfide were dependent on the time of addition of blood to the CytOx assay mixture, and the length of the period during which blood, sulfide, and the enzyme were allowed to equilibrate (Fig. 4). At a given concentration of sulfide, the degree of inhibition of CytOx increased with time of exposure to sulfide ("sulfide equilibration" data in Fig.

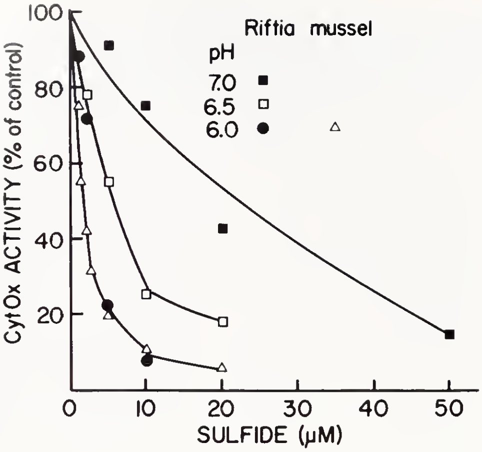

FIGURE 2. Inhibition of CytOx activity of *Riftia* plume tissue and *Bathymodiolus* mantle by sulfide. The pH of assay buffer was varied as indicated. The controls were the activities of the enzyme preparations without addition of sulfide. For *Riftia* CytOx, a twice acid-precipitated preparation was used (Powell and Somero, 1983). For *Bathymodiolus* a crude homogenate was used.

4). When a sulfide-inhibited sample of CytOx was incubated for increasing amounts of time with *Riftia* blood, the reversal of inhibition was enhanced ("blood equilibration" data in Fig. 4). These data indicated that inhibition by sulfide is reversible, and that two equilibria are involved in establishing the effects of sulfide on CytOx: one between sulfide and the enzyme, and one between sulfide and the blood factor that binds sulfide.

The protection afforded by blood (*Riftia*) was a function of pH (Fig. 5). At pH 7.4 the protective effect was much higher than at pH 6.0, a finding which may reflect the pH sensitivity of binding of sulfide to the protective factor in blood (see Discussion). The data in Figure 5 were obtained with blood samples containing different concentrations of sulfide (see legend to Fig. 5). Equilibration of blood with high concentrations

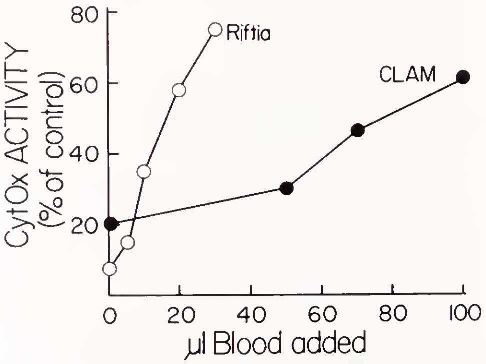

FIGURE 3. Protection by blood of CytOx activity from sulfide inhibition. The line labeled *Riftia* was *Riftia* plume enzyme and blood, and the line labeled clam was *Calyptogena* heart enzyme and blood. The *Riftia* enzyme had been twice acid-precipitated (Powell and Somero, 1983); the clam enzyme preparation was a crude homogenate. The controls were the activities of the enzyme preparations without sulfide added. Sulfide concentration of *Riftia* assays was 5 μM, and of *Calyptogena magnifica* assays was 500 μM.

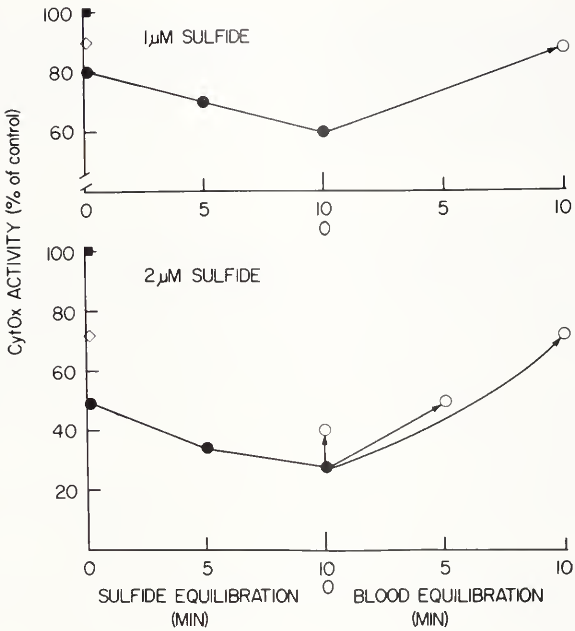

FIGURE 4. Time-dependent effects of sulfide on *Riftia pachyptila* plume CytOx activity, and time-dependent reversal of inhibition by blood. Sulfide was added to CytOx preparations, and activity was measured at the times indicated. The filled circles represent time of pre-incubation of enzyme preparations with sulfide prior to initiation of assay. The open diamond symbols represent addition of 10 μl of blood at t = 0, and immediate assay. Open circles represent addition of blood after 10 min pre-incubation of enzyme preparations with sulfide; assays were then initiated 0, 5, and 10 min after blood addition. Filled squares represent CytOx activity of enzyme preparations without sulfide added.

of sulfide reduced or eliminated the ability of the blood to protect CytOx activity, indicating a saturation of the sulfide-binding capacity of the blood at higher sulfide concentrations. The finding that the protective effects of blood could be reduced or

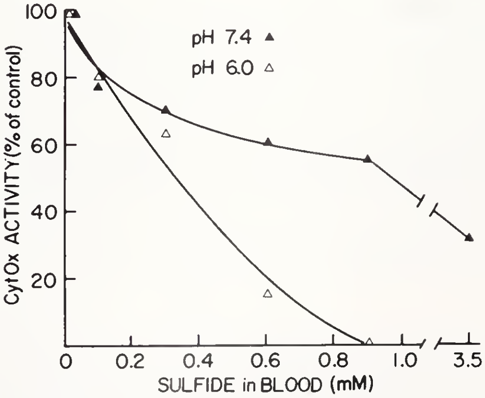

FIGURE 5. Effects of pH and blood sulfide concentration on protection by blood of CytOx activity. Ten μl of blood containing known amounts of sulfide were added to CytOx assays inhibited by 2 μM sulfide (pH 6.0), and 10 μM sulfide (pH 7.4). Sulfide-free blood (10 μl) provided 100% protection (equal to control activity without sulfide added) under the assay conditions used.

eliminated through exposure of the blood to sulfide prior to adding a blood sample to the CytOx assay mixture shows that the protective factors are not oxidizing sulfide, in which case the effects of sulfide would be reduced during equilibration of blood and sulfide. Instead, the protective effects are due to a binding of sulfide that leads to protection of the sulfide from oxidation.

ATP synthesis studies

To test the hypothesis that sulfide and other reduced sulfur compounds are used in these symbioses as important sources of energy, we measured the abilities of sulfide, thiosulfate, and sulfite to stimulate ATP synthesis in freshly prepared tissue homogenates of symbiont-containing and symbiont-free tissues. We used two types of homogenates: ones which were prepared in iso-osmotic buffer (buffered seawater) to avoid osmotic lysis of the bacteria ("intact" studies), and ones prepared in dilute Hepes buffer, in which the bacteria were lysed. Lysis was checked by examining the homogenates microscopically.

The data in Figure 6 show that at least one of the reduced sulfur compounds tested was effective in stimulating ATP synthesis with each species. For homogenates of trophosome, only lysed bacteria were capable of synthesizing ATP, and sulfite and sulfide were the only two reduced sulfur compounds effective in stimulating ATP production. In homogenates of *Calyptogena* gill, only sulfite was able to stimulate ATP production and, as with trophosome, only lysed bacteria were effective in synthesizing ATP under our experimental conditions. Homogenates of gill of *Bathymodiolus* differed in three respects from those of the other species. First, only intact bacteria exhibited ATP production in response to reduced sulfur compounds. Second, thiosulfate was the most effective reduced sulfur compound in driving ATP synthesis. Third, the amount of ATP produced was much lower in *Bathymodiolus* preparations than in the other species.

Stimulation of ATP synthesis by reduced sulfur compounds was not observed for any of the symbiont-free tissues tested (*Riftia* body wall, foot and mantle of *Calyptogena* and *Bathymodiolus;* data not shown).

Bacterial contents of tissues

To determine if the differences in sulfide oxidizing activities and ATP synthesizing capacities were correlated with differences in the contribution of the bacterial endosymbionts to total tissue mass, we counted bacteria in homogenates of the symbiont-containing tissues, measured the sizes and volumes of the bacteria, and computed the percent of tissue mass due to bacteria (Table III). The contribution of bacterial cells to total tissue mass was highest in trophosome, next highest in clam gill, and lowest in the mussel gill, the same ranking found for sulfide oxidizing and ATP synthesizing activities.

DISCUSSION

These studies were designed to elucidate some of the mechanisms by which the three dominant vent animals, *Riftia pachyptila, Calyptogena magnifica,* and *Bathymodiolus thermophilus,* succeed in exploiting the energy of the sulfide molecule while avoiding its toxic effects on aerobic respiration. Our results indicate that the three symbioses share certain mechanisms in common for metabolizing and detoxifying sulfide, yet differ in some aspects of their sulfide relationships.

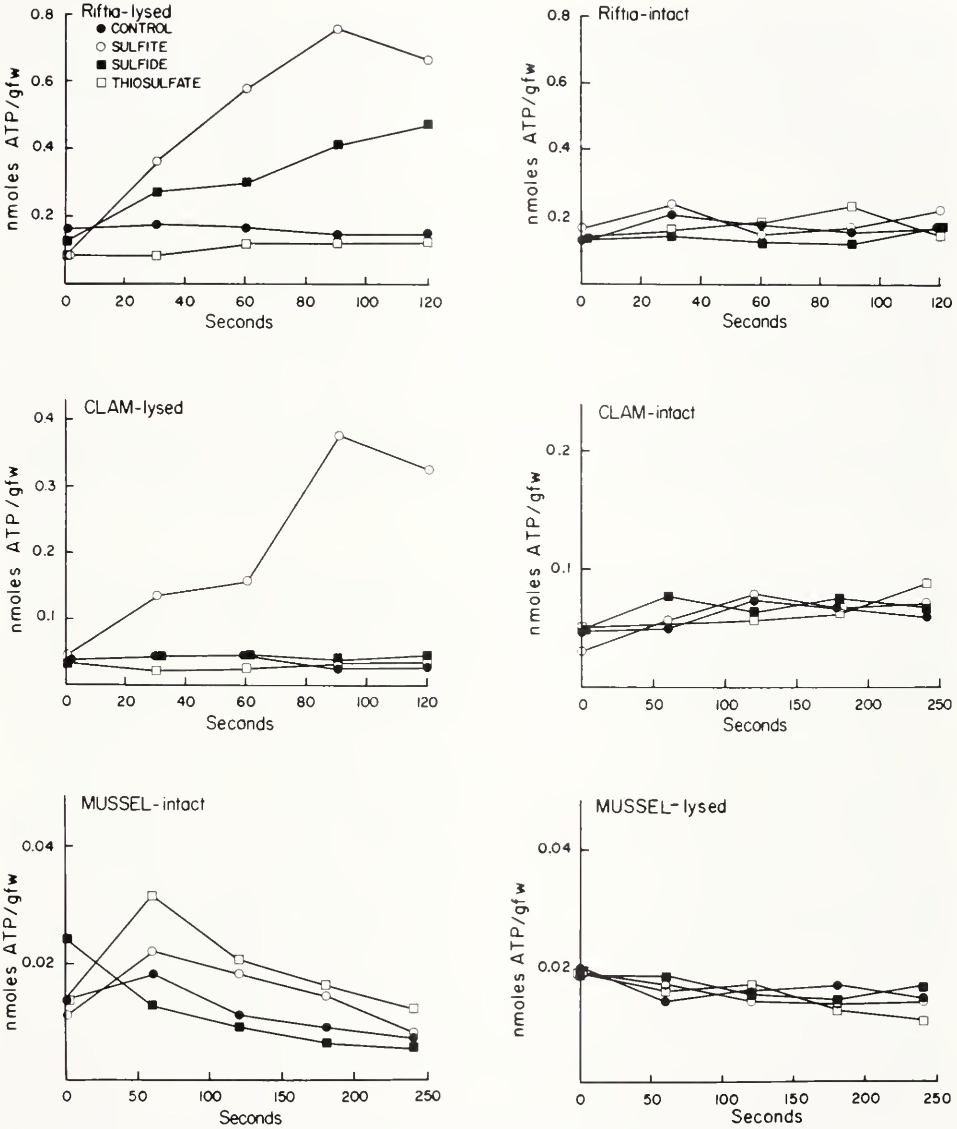

FIGURE 6. ATP concentrations (nmoles ATP/gFW tissue) in homogenates incubated with reduced sulfur compounds [sulfide (■), sulfite (○), or thiosulfate (□)] at 1 m*M* final concentration or no sulfur compound [control (●)]. Lysed samples were homogenized and assayed in low osmolarity buffer; intact samples were homogenized and assayed in buffered artificial seawater. Intactness of bacteria was judged by phase-contrast microscopy. Each frame presents data gathered with a different tissue homogenate. Each symbol represents the average of duplicate samples.

Protection of aerobic respiration from poisoning by sulfide

The occurrence of CytOx activity in all three species (Table II) and the observation that *Riftia* (Childress *et al.*, 1984) and *Calyptogena* (Arp *et al.*, 1984) consume oxygen at rates comparable to those reported for shallow-living species, at comparable mea-

TABLE III

Bacterial composition of symbiont-containing tissues of vent animals

Sample	# Bacteria/gFW	Size of bacteria (μm) (avg, range)	% Bacteria
Riftia pachyptila			
trophosome			
(top)	$4.37 \pm 1.05 \times 10^9$ (3)	4.0, 2–6	15
(bottom)	$10.3 \pm 5.6 \times 10^9$ (3)	4.0, 2–6	35
Calyptogena magnifica			
gill	$3.30–3.64 \times 10^{11}$ (2)	0.75, 0.5–1.0	7.7
Bathymodiolus thermophilus			
gill	$1.70–1.81 \times 10^{11}$ (2)	0.75, 0.5–1.0	4.0

Bacterial composition of tissues. Measurements and calculations are described in Materials and Methods. For *R. pachyptila,* mean ± standard deviation (n) are given, and for the bivalves range and (n) are given.

surement temperatures, suggest that the vent organisms are likely to possess mechanisms for protecting themselves from poisoning by sulfide. The needs for these protective mechanisms are shown by the sensitivities of the CytOx enzymes of these species to sulfide (Figs. 2, 3), sensitivities which are as high as those of CytOx systems of animals from low-sulfide environments (National Research Council, 1979).

Two types of mechanisms for protecting aerobic respiration from poisoning by sulfide were identified in this study. First, in all three species, a "peripheral defense" strategy that employs a zone of sulfide oxidizing activity in cells of the body surface may be important in detoxifying rapidly any sulfide that enters the cells. The histochemical localization studies (Fig. 1) indicate that the sulfide oxidizing activities of body wall muscle of *Riftia* and of foot and mantle tissues of *Calyptogena* (and *Bathymodiolus;* data not shown) are restricted to the superficial cell layer(s) of these tissues. The reported SOx activities in terms of gFW of tissue thus may grossly under-represent the specific activities of SOx in the superficial cell layers of the symbiont-free tissues.

This "peripheral defense" type of sulfide detoxification strategy was previously reported in *Solemya reidi* (Powell and Somero, 1985), and we propose that it may be a generally occurring mechanism in soft-bodied marine invertebrates living in sulfide-rich habitats. For these organisms, there appears to be no impermeable barrier to exclude sulfide from the outer body surface. Arthropods, *e.g.*, the vent crustaceans, appear to lack this "peripheral defense" mechanism, and instead detoxify any sulfide that has entered their circulation by oxidizing it in the hepatopancreas (Vetter *et al.*, 1986). Arthropods may be highly impermeable to sulfide, except at areas where the exoskeleton is very thin, so most of the body surface may not be threatened by sulfide entry and poisoning of respiration.

A second mechanism for preventing poisoning of respiration by sulfide exists in *Riftia* and *Calyptogena.* High molecular weight factors in the bloods of the tube worm (Arp and Childress, 1983; Fisher and Childress, 1984) and the clam (Arp *et al.*, 1984) bind sulfide tightly, and appear capable of extracting sulfide from the environment and transporting it via the circulation to the bacterial symbionts. These binding factors, which are proteins in *Riftia* (Arp and Childress, 1983; Childress *et al.*, 1984) and probably also in *Calyptogena* (Arp *et al.*, 1984) appear to be important in holding free sulfide concentrations in blood to low values. We suggest that as long as the sulfide-binding proteins remain unsaturated, too little free sulfide exists in solution in the blood of these species to pose a significant threat to aerobic respiration.

Bathymodiolus does not have a sulfide-binding protein in its blood (Drs. A. J. Arp and J. J. Childress, University of California, Santa Barbara, pers. comm.), so this type of defense against sulfide poisoning does not play a part in the mussel's strategies for protecting respiration from sulfide.

The role of pH in establishing the effects of sulfide on CytOx activity bears close examination. As shown for CytOx of *Riftia* (Fig. 2), the inhibitory effects of sulfide on CytOx activity increased as pH was reduced. This observation suggests that H_2S, not HS^- or $S^=$, is the inhibitory form of sulfide. A similar conclusion has been reached in studies of other organisms (*cf.*, Environmental Protection Agency, 1976). Near pH 7, roughly equal amounts of H_2S and HS^- are present. When pH is increased above 7, the amount of the inhibitory species, H_2S, falls rapidly and, as shown by our results (Fig. 5) the protection by blood increases as well. The enhanced protection by blood at higher pH values in the physiological pH range is consistent with the findings of Childress *et al.* (1984) that sulfide binding by blood of *Riftia* increased between pH values of approximately 5.5 and 7.5. The finding that sulfide binding by blood and protection by blood of CytOx are high at the average blood pH of *Riftia*, approximately 7.5, (Childress *et al.*, 1984), suggests that protection of respiration by the sulfide-binding protein is highly effective under physiological pH conditions. Intracellular pH values for *Riftia* have not been determined, but it seems reasonable to assume that *Riftia*, like other animals, maintains its intracellular pH approximately 0.4–0.5 pH units acidic to blood (*cf.*, Reeves, 1977). Thus, any free sulfide entering the cells would pose a significant threat to CytOx activity since at pH 7.0–7.1 substantial concentrations of the inhibitory form of sulfide, H_2S, would be present.

Several questions about the mechanisms used to defend against poisoning by sulfide remain. One concerns the means by which sulfide passes from the ambient vent water into the animals' circulatory systems without poisoning mitochondrial respiration. For example, in the plume of *Riftia*, which is thought to serve as the major entry route for sulfide (Childress *et al.*, 1984), large amounts of sulfide must cross a highly aerobic (Table II) tissue. The low sulfide oxidase activities in plume (Table I) suggest that little oxidation of sulfide takes place during the transport process. Oxidation of sulfide during passage across a transport surface into the circulatory system, where it is complexed in a non-toxic form by the sulfide-binding proteins, is undesirable, of course. The maximal energy yield from sulfide will be attained only if sulfide, not some partially oxidized sulfur compound, is delivered to the site of sulfur metabolism, *e.g.*, the bacteria in the trophosome. We suggest that sulfide transport in plume may be pericellular, a route which would avoid the problems of sulfide inhibition of respiration and the partial oxidation of this energy resource.

A second unanswered question about the interactions of aerobic respiration with sulfide concerns the mussel, *Bathymodiolus*, which appears to lack sulfide binding proteins in its blood. The activities of CytOx present in tissues of the mussel may be protected entirely by sulfide oxidase enzymes serving a detoxification role. However, these sulfide oxidizing enzymes could reduce the amount of sulfide reaching the bacterial symbionts (see below).

Reduced sulfur compounds as energy sources

Discussions of the symbioses between sulfide biome invertebrates and their bacterial symbionts have emphasized the potential contributions that reduced sulfur compounds like sulfide might make to the energy needs of these organisms (*cf.*, Felbeck and Somero, 1982; Jannasch and Mottl, 1985). It has been emphasized that carbon dioxide fixation via the Calvin-Benson cycle could be driven by the energy released in bacterial sulfide oxidation, and that the reduced carbon compounds synthesized in the bacterial symbionts could be translocated to the host to satisfy some fraction of its nutritional requirements.

In the clam, *Solemya velum*, Cavanaugh (1983) demonstrated that sulfide and thiosulfate were effective in stimulating carbon dioxide fixation. Belkin *et al.* (1986) showed that sulfide, but not thiosulfate, stimulated the fixation of carbon dioxide in homogenates of *Riftia* trophosome; thiosulfate, but not sulfide, was effective in stimulating carbon dioxide fixation in homogenates of gill from *Bathymodiolus*. To our knowledge, these are the only studies that have demonstrated directly the roles of reduced sulfur compounds in driving endergonic processes in these symbioses.

To gain additional understanding of the efficacy of reduced sulfur compounds in supplying energy for these symbioses, we reasoned that it would be especially important to determine how these compounds affected the ATP synthesis of the organisms. Because ATP is the dominant "energy currency" of the cell, changes in ATP concentrations in response to exposure to different reduced sulfur compounds could be a very sensitive indicator of the importance of these compounds to the generation of biologically useful forms of energy in the symbioses. Prior to our studies the capacities of these symbioses to trap the energy released during the oxidation of reduced sulfur compounds in the form of ATP had not been investigated. Neither had there been study of the relative abilities of different reduced sulfur compounds, *e.g.*, sulfide, sulfite, and thiosulfate, to supply energy in these symbioses.

The results of our experiments suggest that the symbiont-containing tissues of *Riftia*, *Calyptogena*, and *Bathymodiolus* can exploit the energy of reduced sulfur compounds. Because we used unfractionated homogenates in our studies, *i.e.*, animal tissue and bacteria were present, our results do not demonstrate unambiguously that the symbionts were solely or primarily responsible for the ATP production driven by reduced sulfur compounds. The correlation between ATP production and bacterial contribution to tissue mass is consistent with a dominant role for the symbionts in this process (Table III); however, a role for animal-localized ATP synthesis mechanisms cannot be excluded (see below).

The reduced sulfur compounds most effective in driving ATP synthesis differed among the three symbioses (Fig. 6). Also, the total amount of ATP that was produced by oxidation of reduced sulfur compounds differed among the three symbioses studied (Fig. 6). In *Riftia* trophosome homogenates, sulfide and sulfite were most effective in stimulating ATP production, while in *Calyptogena* only sulfite was effective, and in *Bathymodiolus*, thiosulfate was most effective (Fig. 6). In *Riftia* and *Calyptogena*, only homogenates containing lysed bacteria exhibited the capacity to exploit reduced sulfur compounds, while in *Bathymodiolus* intact bacteria had to be present for ATP synthesis to occur. *Riftia* trophosome had the highest ATP synthesizing capacity, followed by the gills of *Calyptogena* and *Bathymodiolus*.

The finding that sulfide was effective only in the case of *Riftia*, where the highest activities of SOx were found, and where SOx was clearly localized in the bacterial symbionts (Fig. 1), suggests that the tube worm may be the only one of these three symbioses to supply sulfide directly to its bacterial symbionts.

In the two bivalves, where sulfide was not effective as an energy source for driving ATP production under our experimental conditions, the abilities of sulfite and thiosulfate to stimulate ATP synthesis suggest that only oxidation products of sulfide may be delivered by the animal to its symbionts. The sulfide oxidizing activities found in the gills of the two vent bivalves could not be localized histochemically due to the very small sizes of the bacteria (Table III). Therefore, it is not clear whether the measured SOx activities are animal or bacterial. However, the finding that in gills of *Solemya reidi* all of the SOx activity is found in the animal compartment (Powell and Somero, 1985) is an interesting precedent in this context. Perhaps in the vent bivalves as well the initial step(s) in sulfide oxidation occur in the animal tissue.

Belkin *et al.* (1986) found sulfide to be the most effective energy source for driving net CO_2 fixation in trophosome of *Riftia*, and thiosulfate to be the most effective energy source in *Bathymodiolus*. Our findings on the relative abilities of reduced sulfur compounds to drive ATP synthesis agree with their results, and we suggest oxidation of sulfide by the animal tissues as the source of thiosulfate for the symbionts of *Bathymodiolus*.

If in the bivalves the initial step(s) in sulfide oxidation occur in the animal compartment of the symbiosis, not in the bacteria, then the possibility exists that mitochondrial ATP production might be driven by the energy released in sulfide oxidation. This is the case for mitochondria of *Solemya reidi* (Powell and Somero, 1986). Because the homogenization procedures we used in the studies of the three vent symbioses would have ruptured mitochondria, we cannot exclude the possibility that mitochondria in both symbiont-containing and symbiont-free tissues are able to exploit the energy released in mitochondrial sulfide oxidation to drive ATP synthesis. We plan to examine this possibility during future studies of the vent animals.

In conclusion, our studies show that, despite many similarities, the three symbioses studied also exhibit important differences, *e.g.*, in preferred reduced sulfur compounds for driving ATP synthesis, in the absolute capacity for ATP synthesis in the symbiont-containing tissues, and in mechanisms for transporting and detoxifying sulfide. The three symbioses may also differ in the relative roles played by the bacterial symbionts in the nutritional needs of the animal. This is suggested by both anatomical and biochemical results. *Riftia* entirely lacks a digestive system (Jones, 1981), and the unusual carbon isotope ratios of its tissues, ratios which are the same in symbiont-containing and symbiont-free tissues (Rau, 1981), suggest a strong reliance on the reduced carbon compounds translocated from bacterial to animal cells. *Calyptogena* has a greatly reduced digestive system (Boss and Turner, 1980), and has more bacterial endosymbionts per mass of gill than *Bathymodiolus*. The activities of SOx and ATP synthesis are higher in the clam than in the mussel as well. *Calyptogena* is found exclusively at sites of active venting (see Arp *et al.*, 1984), whereas *Bathymodiolus* is found in active venting sites and at sites peripheral to the main venting regions (see Hessler and Smithey, 1983). *Bathymodiolus* has a well-developed digestive system, and a ciliary-mucus net feeding capacity (Kenk and Wilson, 1985). Thus, where *Riftia* and *Calyptogena* may depend absolutely on a symbiotic source of reduced carbon compounds, *Bathymodiolus* may be able to exploit a greater variety of microhabitats in the vent ecosystem.

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MOLECULAR BIOLOGY OF THE EARLY MOUSE EMBRYO

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ABSTRACT

The transition from maternal to embryonic control of development in the early mouse embryo occurs during the 2-cell stage. By the 2-cell stage, all classes of RNA are transcribed from the embryonic genome. Most of the changes in protein synthetic pattern that occur during the first cleavage are post-transcriptionally regulated. Later events, including compaction and blastocyst formation, require transcription from the embryonic genome, but some elements of post-transcriptional regulation are also involved. The result of the preimplantation developmental period is the formation of the blastocyst with two distinct cell types, the trophectoderm and inner cell mass cells. Each cell type is committed to a different developmental pathway and exhibits specific patterns of protein synthesis and DNA methylation.

INTRODUCTION

In the past decade, significant progress has been made in the manipulation of reproduction and development in mammalian species. Artificial insemination and embryo transfer are now widely used in the livestock industry and in the treatment of human infertility. While the length of time a mammalian embryo spends in the preimplantation period and the cell number achieved prior to implantation varies from one species to another, early development in every case is characterized by the formation of a blastocyst containing two morphologically and biochemically distinguishable cell types. On this basis the mouse is a convenient and inexpensive model for basic research. Moreover, genetic uniformity and the ability to use genetic approaches are important considerations in any biological study. This consideration is satisfied in mice by the many inbred strains or F1 hybrids available.

The acquisition of the background knowledge and technical achievements (see Daniel, 1971; Daniel, 1978) necessary for experimental manipulation of mammalian eggs and embryos has largely occurred within the mouse system. For example, it was in the mouse that it was determined that early blastomeres are totipotent (Tarkowski and Wroblewska, 1967) and that parthenogenetically activated eggs can develop up to post-implantation stages (Graham, 1970; Tarkowski *et al.,* 1970; Surani and Barton, 1983). Similarly, the first reliable *in vitro* culture methods (Brinster, 1963; Whittingham, 1971) and embryo freezing and thawing techniques (Whittingham *et al.,* 1972) were developed for mouse embryos. Successful constructions of chimaeric mouse embryos of tetraparental heritage have been included in the developments (Mintz, 1965). Other experimental manipulations for nuclear transfer (Surani and Barton, 1983; McGrath and Solter, 1984) and gene transfer by microinjection (Gordon and Ruddle, 1981; Costantini and Lacy, 1981; Wagner *et al.,* 1981) have also been pioneered in the mouse system. Elegant experiments with appropriate gene constructs to study expression of exogenously supplied genes ("transgenes") have followed (Palmiter *et al.,* 1982; Krumlauf *et al.,* 1985). All of these recent developments have

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depended on general knowledge about macromolecular synthesis and gene expression in the egg and early embryo.

This review begins with an outline of general procedures for handling mice, recovering early embryos, and assessment of the major morphological changes accompanying development to the blastocyst stage. An evaluation of maternal control of early development is made prior to consideration of transcription from the embryonic genome. Patterns of protein synthesis influenced by post-transcriptional processes during maternal control of the first cleavage are included along with a summary of protein synthetic activity dependent upon transcription of template RNAs from the zygote genome. The review ends with a discussion of the appearance of cell-type specific patterns of protein synthesis and DNA methylation at the blastocyst stage. Where possible, relevant comparisons to parallel developmental features in eggs and embryos of the well-studied marine organisms and other comparative systems have been included.

OBTAINING EGGS AND EMBRYOS

Successful embryological studies, especially at the molecular level, require a large supply of eggs and embryos. In outbred strains of mice, natural cycles and mating lead to the generation of 12 to 14 embryos per female. Inbred strains, which are less vigorous in reproductive capacity, have smaller litter sizes. Hence, in many laboratories, female mice are hormonally stimulated to cause an increase in the number of eggs that are ovulated. Hormonal manipulation also confers the further advantage of being able to regulate the time of ovulation (within certain limits) such that the collection of eggs or embryos at specific stages of development coincides with work schedules and convenience of the investigator.

Procedures for the superovulation of mice and the timing of ovulation and mating have been described by Gates (1971) and Whitten and Champlin (1978), respectively. The highest yield of eggs (average of 89.5 per female; Gates, 1971) is obtained when prepuberal females of three weeks of age are superovulated, because this is a time when a wave of follicle maturation is occurring at the ovarian level. The immature mouse cannot, however, be used to derive embryos that have developed *in vivo* beyond the 2-cell stage (*e.g.*, morulae or blastocysts). Normal preimplantation development probably does not occur because eggs and embryos are transported too rapidly through the oviduct to the uterus (Gates, 1971). Fertilized eggs and 2-cell embryos from appropriate strains of prepuberal females will develop to blastocysts (with normal attrition rates) if subjected to *in vitro* culture. Because of high numbers at the outset, this can be a good source of *in vitro*-derived material. In all superovulation procedures, a larger proportion of preimplantation embryos (about 12%) have sister-chromatid exchange chromosomal abnormalities than do embryos (about 3.5%) derived from normal matings (Elbling and Colot, 1985).

To obtain embryos developed *in utero,* many investigators use random-bred mice that have reached reproductive age at 7 to 8 weeks. Superovulation at this stage leads to egg yields (30 to 35) that are approximately three times that of normal cycling females. The females are stimulated by intraperitoneal injection of 5 to 10 I.U. of pregnant mare serum gonadotrophin (PMSG). This preparation has a relatively stable half-life (and therefore requires only a single injection to maintain stimulation of follicles over the 2½ day period to ovulation) and is a relatively inexpensive source of FSH (follicle-stimulating hormone) activity. Induction of ovulation is stimulated 44 to 48 hours later by the intraperitoneal injection of 5 to 10 I.U. of hormone with LH (luteinizing hormone) activity. The usual commercial source is human chorionic gonadotrophin (HCG). Rupture of matured Graafian follicles and release of ova to

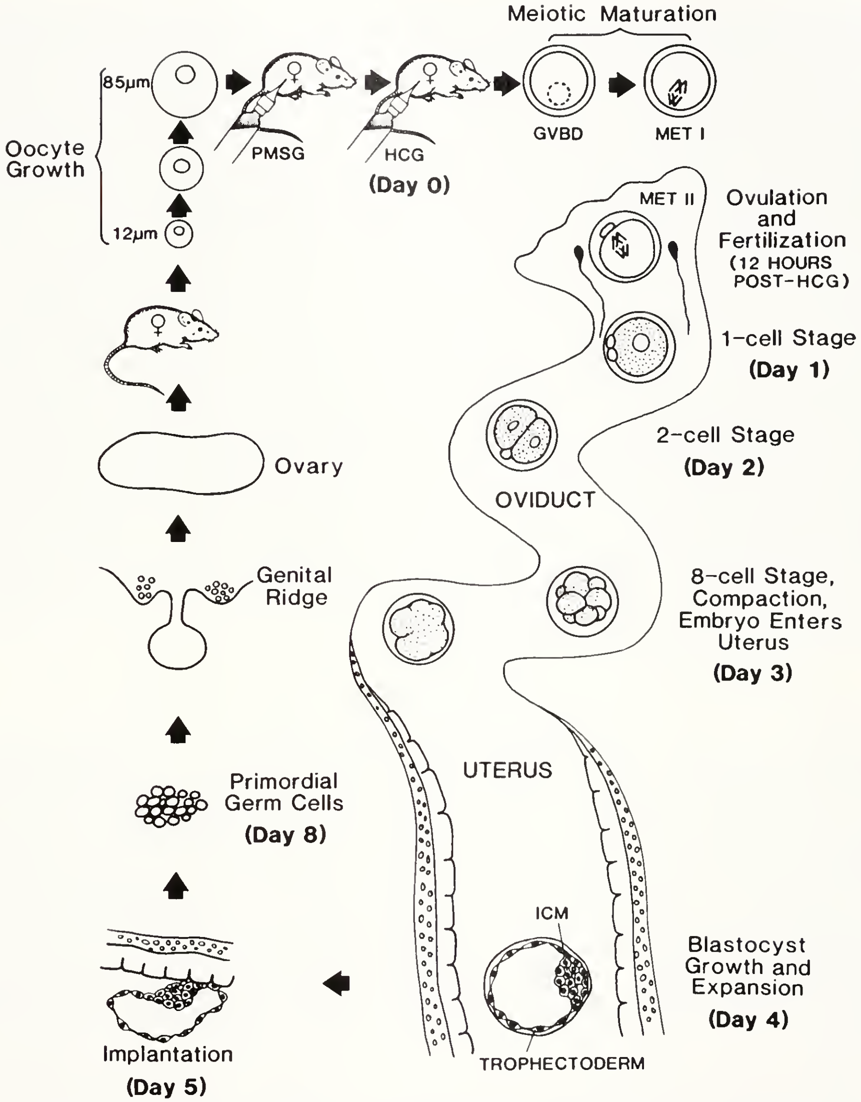
Meiotic Maturation
85µm
12µm
Oocyte Growth
PMSG
HCG
(Day 0)
GVBD
MET I
MET II
Ovulation and Fertilization (12 HOURS POST-HCG)
1-cell Stage (Day 1)
2-cell Stage (Day 2)
Ovary
OVIDUCT
Genital Ridge
8-cell Stage, Compaction, Embryo Enters Uterus (Day 3)
UTERUS
Primordial Germ Cells (Day 8)
ICM
Blastocyst Growth and Expansion (Day 4)
Implantation (Day 5)
TROPHECTODERM

FIGURE 1. Diagrammatic outline of early development in the mouse. Abbreviations are as follows: PMSG, pregnant mare serum gonadotrophin; HCG, human chorionic gonadotrophin; GVBD, germinal vesicle breakdown; MET I, first meiotic metaphase; MET II, second meiotic metaphase; ICM, inner cell mass.

the fimbrial end of the oviduct occurs approximately 12 hours after HCG administration (Fig. 1).

The light-dark sequence is a major modulator in polyestrous mammals like mice. Endogenous LH surges lead to an ovulation time about 3 h after the mid-point of the dark period (Whitten and Champlin, 1978). Hence, it is common to employ a system in which the mouse room is maintained with 12 to 14 hours of light and 10 to 12 hours of dark with lights on at 5:00 to 7:00 hours and lights off at 19:00 hours. Injections of PMSG are often scheduled between 16:00 to 20:00 hours, and HCG is administered at 12:00 to 16:00 hours two days later. A 4:00 p.m. (16:00 hours) HCG injection leads to ovulation at about 4:00 a.m., three hours after the mid-point of a 7:00 o'clock to 7:00 o'clock dark cycle, and coincides with any endogenous LH surge. If females are placed with fertile active males at the time of the HCG injection, copulation occurs and fertilization of eggs takes place in the fimbrial end of the oviduct shortly after ovulatory release. Of course, if it is desirable to study ovulation and fertilization events during normal working hours, the cycle can be delayed by changing the dark-light cycle of the mouse room and the injection schedule.

The presence of a copulation or vaginal plug (a coagulation of seminal proteins) on the morning after mating is the criterion commonly used to infer that fertilization occurred and that preimplantation embryogenesis was initiated. With hormonal manipulation of females and selection of reproductively active males, plug rates of 75 to 80% are commonly achieved. This aids the investigator in obtaining sets of staged pregnant females from which to derive embryos at a particular stage of development. Random matings of spontaneously cycling females invariably yield low plug rates. Yet, a peak of fertile mating (up to 50% of females) often takes place on the third night after females are paired with males, due to acceleration of estrous cycling under the influence of male pheromones (Whitten and Champlin, 1978). Alternatively, females in proestrous can be identified by microscopic examination of cells from vaginal smears of mice or, for the experienced eye, the changes in vaginal size and coloration (Champlin *et al.,* 1973). These pre-selected females from a natural cycling population will yield high plug rates upon mating.

TIME COURSE OF MORPHOLOGICAL CHANGES DURING PREIMPLANTATION DEVELOPMENT

During early embryonic life of mice destined for production of preimplantation embryos, primordial germ cells arise and migrate via the hindgut and dorsal mesentery to the dorsal body wall and the genital ridges (Fig. 1). In female embryos oogonia transform into oocytes, which progress through early meiotic prophase during fetal life and reach the diplotene stage at about the time of birth. During prepuberal and reproductive life, sets of oocytes begin to grow from a diameter of about 12 μm to a diameter of about 85 μm over a two-week period (Fig. 1). During this period, the acellular zona pellucida that surrounds the plasma membrane of the ovulated egg (Fig. 2b) is laid down (Bleil and Wassarman, 1980). Under appropriate hormonal influence, these oocytes can be induced to undergo meiotic maturation and to be ovulated.

At the time of ovulation, the unfertilized mouse egg, like that of *Xenopus,* is arrested at second meiotic metaphase and has extruded one polar body (Fig. 2b). This state differs from that in sea urchin eggs which have completed meiosis prior to fertilization as well as the situation in *Spisula* and *Asterias,* where eggs are arrested at first meiotic prophase at the time of sperm penetration (Browder, 1984). Within 1 to 3 hours after fertilization, mouse eggs complete meiosis and generate a second polar body (Fig. 2c). The sperm head decondenses to form the male pronucleus 4 to 8 hours after sperm entry, and the female pronucleus forms between 5 to 9 hours after sperm penetration (Howlett and Bolton, 1985). The pronuclei move to the center of the egg, DNA replication occurs, pronuclei fuse, and the first cleavage division takes place about 20 hours after the time of sperm entry (Howlett and Bolton, 1985) or roughly 32 to 34 hours after the program is initiated by administration of HCG to induce ovulation (Fig. 1). Subsequent cleavages occur at roughly 12-hour intervals but synchrony among blastomeres is lost early. It is interesting to note that the first polar body often degenerates during the first cell cycle. The second polar body becomes aligned in the plane of the first cleavage furrow (Howlett and Bolton, 1985) and is nearly always positioned between the two blastomeres at the 2-cell stage (Fig. 2d and 2e).

Following successive cleavages to the 8-cell stage (Fig. 2h), a morphological reorganization of the embryo called compaction (Fig. 2i) occurs at about 72 hours post-HCG administration. The distinct outline of individual blastomeres is lost and cells flatten tightly against one another. Gap junctions form in concert with the tight intercellular contacts and membrane and other cellular components rearrange from a nonpolarized to a radially polarized pattern (Ducibella and Anderson, 1975; Ducibella *et al.,* 1977; Lo and Gilula, 1979; Ziomek and Johnson, 1980; Reeve and Ziomek, 1981). The development of cell contacts fixes cells in position and the polarity which occurs during compaction establishes an "inside" and "outside" orientation to blastomeres. Polarity is maintained as cell division continues in the embryo and the embryo is transported from the oviduct to the uterus (Fig. 1). Cells of the embryo tend to differentiate along different lineages depending upon their relative position as "inside" or "outside" in the compacted morula. Within the 64- to 128-cell blastocyst (fourth day of development), an outer layer of trophectoderm cells surrounds the inner cell mass (ICM) cells in the blastocyst cavity (Fig. 2l). Just prior to implantation, the ICM differentiates into primitive endoderm and primitive ectoderm. The trophectoderm and primitive endoderm gives rise to extraembryonic structures and the embryonic contribution to the placenta, whereas the primitive ectoderm becomes the embryo proper (Papaioannou, 1982; Gardner, 1982).

Maternal Messenger RNA Utilization After Fertilization

During oocyte development in the mouse, all classes of RNA are synthesized and accumulated in the egg. Polyadenylated RNA [poly(A)+ RNA], as a marker of putative mRNA, also accumulates and has a high stability. The information available on RNA and protein synthesis during oogenesis in the mouse will not be reviewed here because it has been covered recently in other publications (Bachvarova, 1985; Schultz, 1986). Suffice it to say that during oocyte growth and meiotic maturation, several changes in the two dimensional patterns of newly synthesized protein occur, and during meiotic maturation about half of the accumulated polyadenylated RNA becomes either deadenylated or degraded (Bachvarova, 1985). The newly ovulated mouse egg contains about 23 ng of proteins (Brinster, 1967), between 0.35 to 0.50 ng of total RNA (Olds *et al.,* 1973; Piko and Clegg, 1982), and about 20 pg of poly(A)+ RNA (Piko and Clegg, 1982).

The first cleavage of the mouse zygote is controlled largely, if not exclusively, by informational macromolecules accumulated in the egg. From the 2-cell stage onward, transcription from the zygote genome is necessary for normal development to continue. These conclusions are derived from several lines of experimentation. First, 30 to 40% of the bulk of maternal RNA (Bachvarova and De Leon, 1980), 70% of the total poly(A)+ RNA (Levey *et al.,* 1978; Piko and Clegg, 1982), and as much as 90% of the histone (Graves *et al.,* 1985a) and actin (Giebelhaus *et al.,* 1985) messenger RNA is degraded within the first 24 hours after fertilization as development proceeds to the 2-cell stage. In this regard it is interesting to note that exogenous globin mRNA injected into newly fertilized mouse eggs is actively translated 15 to 17 hours later but is also eliminated on a functional basis by the 4-cell stage (Brinster *et al.,* 1980). Second, while some RNA synthesis has begun within pronuclei of the mouse zygote, the rate of RNA synthesis increases markedly up to the late 2-cell stage such that the synthesis of all classes of RNA is readily detectable (Young *et al.,* 1978; Piko and Clegg, 1982; Clegg and Piko, 1983a, b). Third, a large number of changes in the

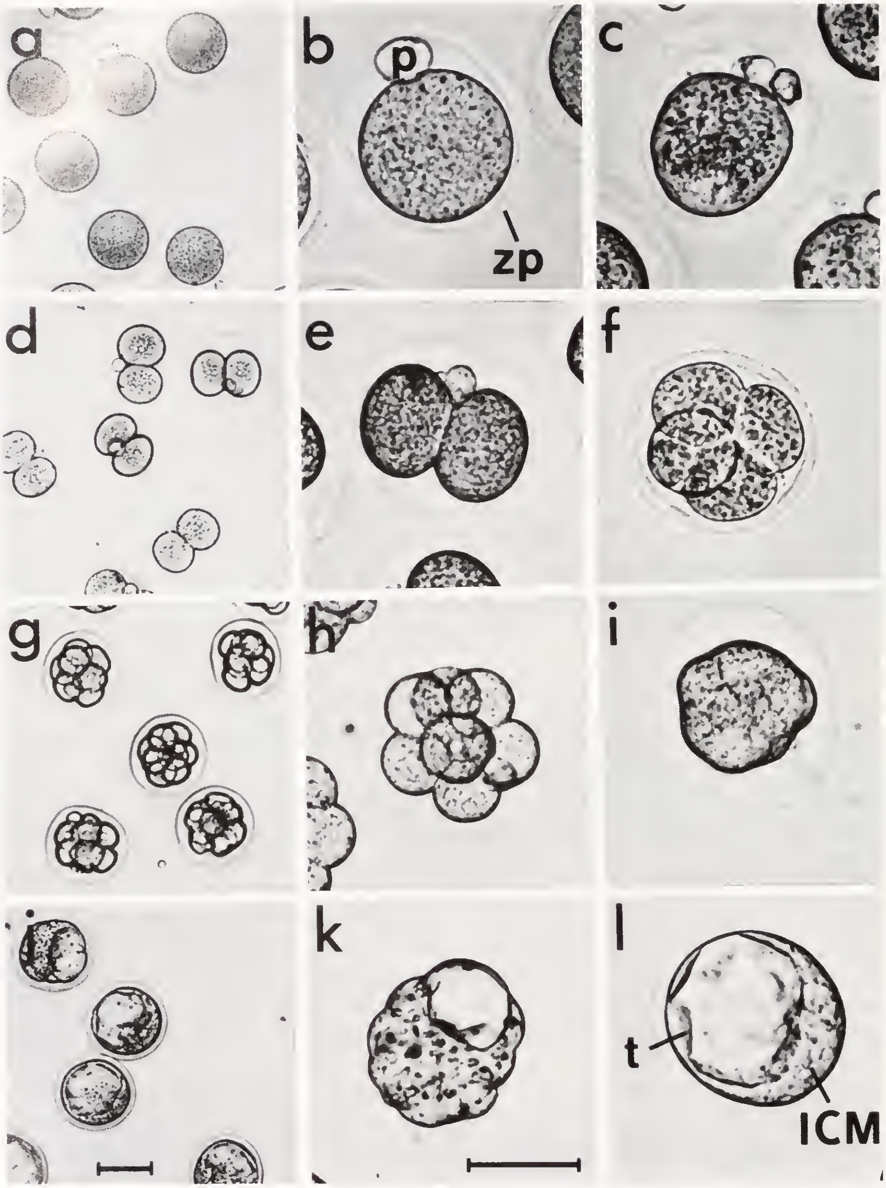

FIGURE 2. Photomicrographs of mouse eggs and preimplantation stages of development. Panels a, d, g, and j show low power views: a, unfertilized eggs at 16 hours post-HCG; d, 2-cell embryos at 42 hours post-HCG; g, uncompacted 8-cell embryos at 66 hours post-HCG; and j, blastocysts at 96 hours post-HCG. The scale bar in j is 50 μm. For higher power photographs, the scale bar in k represents 50 μm. Stages are as follows: b, newly ovulated unfertilized egg with one polar body at 14 hours post-HCG; c, fertilized egg with two polar bodies at 18 hours post-HCG; e, 2-cell embryo at 42 hours post-HCG; f, 4-cell stage at 54 hours post-HCG; h, uncompacted 8-cell embryo at 66 hours post-HCG; i, compacted 8-cell embryo at 72 hours post-HCG; k, early cavitating blastocyst at 90 hours post-HCG; l, mid-blastocyst stage at 98 hours post-HCG. Abbreviations are as follows: p, polar body; zp, zona pellucida; ICM, inner cell mass; t, trophectoderm. All photomicrographs were taken with a Zeiss IM-35 inverted phase contrast microscope.

polypeptide synthetic pattern occur as fertilized eggs develop to the 2-cell stage (van Blerkom and Brockway, 1975; Levinson *et al.,* 1978; Braude *et al.,* 1979; Howe and Solter, 1979; Cullen *et al.,* 1980). These changes appear to be regulated exclusively at the post-transcriptional level utilizing maternal components, since they also occur in physically enucleated eggs (Petzholdt *et al.,* 1980) or in eggs treated with the transcriptional inhibitor α-amanitin (Braude *et al.,* 1979; Flach *et al.,* 1982; Bolton *et al.,* 1984). Fourth, fertilized eggs treated with transcriptional inhibitors like α-amanitin and actinomycin D are markedly inhibited in development beyond the 2-cell stage (Golbus *et al.,* 1973; Warner and Versteegh, 1974). Finally, genetic variants of proteins have been used to advantage to identify paternal gene expression in embryos derived from crosses of appropriate mouse lines. Paternally derived β-glucuronidase (Wudl and Chapman, 1976) and β_2-microglobulin (Sawicki *et al.,* 1982) are both detectable by the late 2-cell stage. Since spermatozoa do not contribute mRNA to the egg, this genetic evidence, along with the biochemical studies already listed, confirms that some newly derived transcripts from the embryonic genome are utilized at least by the 2-cell stage.

Taken together, the work presented above indicates that the transition from maternal to embryonic control in the mouse embryo occurs at the 2-cell stage. The time course of this first cell division covers approximately 24 hours. In the same period of time, a fertilized *Xenopus* egg, under normal conditions, would have developed to the 10,000-cell gastrula stage and a sea urchin embryo would have developed to a 500-cell hatching blastula stage (Davidson, 1976). Similarly, the period of maternal control extends to the gastrula stage in *Xenopus* embryos and to the blastula stage in sea urchin embryos (Davidson, 1976). It is possible that the long cell-cycle for the first division in the mouse is sufficient to allow chromatin re-organization, new transcription, and early transition of genetic control from maternally derived to zygote genome-derived messenger RNA molecules.

Biochemical characterization of the maternal RNA in sea urchin and *Xenopus* eggs has revealed that as much as 70% of the mass of poly(A)+ RNA contains interspersed repetitive sequence elements (Costantini *et al.,* 1980; Anderson *et al.,* 1982). These molecules are large (often 5 to 15 kilobases in length) and similar in structure to nuclear RNA. Similar transcripts are not present in the poly(A)+ mRNA associated with polysomes of later gastrulae stages of development. This RNA with interspersed repeats is not functional when added to *in vitro* translation systems (Richter *et al.,* 1984). Analysis of poly(A)+ RNA in the mouse egg, however, suggests the absence of such non-functional transcription products containing interspersed repetitive elements (Kaplan *et al.,* 1985). One of the major repetitive elements in human and mouse DNA is the Alu sequence family (Jelinik and Schmid, 1982). RNA from full-grown oocytes and ovulated eggs does not contain any larger proportion of sequences complementary to Alu repetitive elements than does poly(A)+ RNA from differentiated cell types such as liver and brain (Kaplan *et al.,* 1985). Moreover, the molecular weight distribution of molecules containing sequences complementary to Alu DNA probes is similar in mouse egg, liver, and brain cytoplasmic RNA, a finding one would not expect if there were large unprocessed RNA transcripts in mouse egg cytoplasm (Kaplan et al., 1985).

Fertilization of the sea urchin egg triggers a series of events that results in a marked increase in the rate of protein synthesis within only a few minutes of sperm entry (Denny and Tyler, 1964; Epel, 1967). Part of this is mediated by mobilization of stored maternal mRNA components (Winkler *et al.,* 1985) into polysomes (Monroy and Tyler, 1963) and part by an increase in translation activity and elongation rate (Brandeis and Raff, 1979; Hille and Albers, 1979; Winkler *et al.,* 1985). Accompanying the increase in protein synthetic rate during the first cleavage is the doubling of the amount of poly(A)+ RNA due to cytoplasmic polyadenylation of pre-existing mRNA species (Slater *et al.,* 1973; Wilt, 1973). The significance of this event is unknown since the rise in protein synthetic rate occurs even when cytoplasmic polyadenylation is blocked in sea urchin embryos treated with cordycepin (Mescher and Humphreys, 1974).

When [^{3}H]-adenosine is used as a precursor, a higher rate of incorporation of adenosine into poly(A) tails *versus* internal locations in the RNA of the 1-cell mouse embryo is observed (Young and Sweeney, 1979; Clegg and Piko, 1983a, b). This cytoplasmic polyadenylation process results in about a 40% increase in the number of poly(A)+ RNA molecules in the newly fertilized 1-cell zygote compared to the ovulated egg (Clegg and Piko, 1983a, b). The net increase involves polyadenylation of previously stored non-adenylated RNA molecules, but there is also a component involving degradation of some pre-existing poly(A)+ RNA. For example, actin mRNA is partially deadenylated in fertilized eggs (Bachvarova *et al.,* 1985). Thus, there appears to be a turnover in the poly(A)+ RNA population in the 1-cell embryo and this could lead to changes in the amounts of individual mRNAs and, in part, to the observed changes in protein synthetic pattern that occur in the 1-cell to 2-cell transition period. As documented earlier, 70% or more of the egg poly(A)+ RNA is eliminated through turnover by the late 2-cell stage (Piko and Clegg, 1982; Clegg and Piko, 1983a).

Post-transcriptional modification of maternal mRNA through capping has also been studied. Addition of [^{2}H]-methyl cap structures to maternally derived histone mRNAs following fertilization of the sea urchin egg has been reported (Caldwell and Emerson, 1985). The mRNAs coding for these early cleavage type histones are stored in the female pronucleus (De Leon *et al.,* 1983) and have no access to the translational machinery of the egg until released after the first nuclear division. The capping process accompanies the translation of these histone mRNAs. It has been suggested that capping of maternal mRNA may be a mechanism by which stored mRNA in sea urchin eggs is activated because it has been shown that, contrary to mammalian cells, sea urchin eggs lack the ability to initiate translation of uncapped mRNAs and have an absolute requirement for 5′ cap structures (Winkler *et al.,* 1983).

The incorporation of a low level of [^{3}H]-guanosine into 5′-terminal m^{7}G structures of RNA in 1-cell mouse embryos has also been reported (Young, 1977). Since more radioactivity was observed in m^{7}G structures than in Gp derived from internal positions, it was postulated that capping of pre-existing RNA molecules may occur in newly fertilized mouse eggs (Young, 1977). In other studies which assessed the degree of capping by translational inhibition with cap analogues and end-labeling of mRNA molecules after enzymatic removal of cap structures with tobacco acid phosphatase, no differences in unfertilized and fertilized egg mRNA were observed (Schultz *et al.,* 1980). Although post-transcriptional regulation of maternal mRNA in the 1-cell mouse embryo via capping is an appealing proposal, such a mechanism should be viewed with caution since uncapped mRNAs in mammalian cells are unstable (Banerjee, 1980) and since cap structures are required on pre-mRNAs of mammalian cells for correct splicing and excision of intervening sequences (Konarska *et al.,* 1984; Kramer *et al.,* 1984).

RNA Synthesis During Preimplantation Development

Early attempts to measure incorporation of [^{3}H]-uridine into RNA in the 1-cell mouse embryo yielded inconclusive results. Low levels of incorporation were observed, but the significance of the findings was complicated by the fact that failure to detect RNA polymerase activity in 1-cell mouse embryos (Moore, 1975) suggested that the embryonic genome was not active until after the first cleavage. In studies on absolute rates of synthesis based on specific activities of precursor pools, Clegg and Piko (1977) discovered that [^{3}H]-adenosine was taken up and converted to ATP in mouse embryos about 1000 times more readily than the parallel conversion of uridine to UTP. Using [^{3}H]-adenosine as a labeled precursor, experiments to re-examine RNA synthesis in the 1-cell embryo were conducted (Clegg and Piko, 1983a, b). Some [^{3}H]-adenosine was observed to be incorporated into tRNA in the 1-cell embryo, but the majority was due to turnover of the 3′-terminal AMP (Clegg and Piko, 1983b). The synthesis of some heterogeneous RNA devoid of poly(A) tracts was also observed, but at a low rate (0.3 pg·cell/h). There was also a low rate of synthesis of internally labeled poly(A)+ RNA but the majority of [^{3}H]-adenosine incorporation in this class of RNA molecules was associated with turnover of 3′-terminal poly(A) tails (Clegg and Piko, 1983a, b). By the 2-cell stage, the rate of poly(A)+ RNA synthesis was measured to have increased five-fold over the 1-cell rate and ribosomal RNA synthesis was occurring at the rate of 0.4 pg/embryo/h (Clegg and Piko, 1983b).

The capacity for transcription of RNA polymerase II genes in the 1-cell fertilized egg is demonstrated conclusively by plasmid microinjection experiments. Both the herpes simplex virus (HSV) thymidine kinase (TK) gene and a hybrid gene in which the HSV TK gene is fused to a mouse metallotheionein promotor are transcribed and expressed as enzymatic activity (translation) in fertilized mouse eggs (Brinster *et al.*, 1982). In contrast to RNA polymerase type III genes (Brinster *et al.*, 1981), the TK genes are transcribed much more effectively in fertilized eggs than in growing oocytes and other pre-fertilization stages (Brinster *et al.*, 1982; Chen *et al.*, 1986). An interesting observation on RNA processing also emerges from these studies. By comparison of expression of the HSV-TK gene (no introns), the SV40 TK gene (one intron), and the chicken TK gene (six introns), the presence of introns is associated with decreased expression (Chen *et al.*, 1986). This may reflect a limited capacity for RNA splicing at this early stage of development. Nonetheless, the experiments demonstrate an enhanced capacity for transcription in fertilized eggs compared to the unfertilized ovum.

On the basis of specific activities of uridine pools, Clegg and Piko (1977) measured the absolute rates of RNA synthesis from the 2-cell stage to the blastocyst and observed a fifty-fold increase on an embryonic basis. However, if rates are calculated on a cellular basis, the value changes more modestly from about 1.25 pg/cell/h at the 2- to 4-cell stage, to 2.5 pg/cell/h in the 8-cell embryo to about 5 pg/cell/h in the blastocyst. These rates are not very much lower than the rate of total RNA synthesis (5.7 pg/cell/h) reported for exponentially growing HeLa cells (Brandhorst and McConkey, 1974). The rates are greater than those measured for heterogeneous RNA synthesis in cleavage and blastula stages of sea urchin embryos (Brandhorst and Humphreys, 1971; Wu and Wilt, 1974). In summary, all classes of RNA, including poly(A)+ RNA, are actively synthesized from the 2-cell stage and onwards throughout the preimplantation period (Levey *et al.*, 1978; Piko and Clegg, 1982).

Whereas maternal oocyte mRNA is largely degraded by the late 2-cell stage (see previous section), turnover rates of newly synthesized RNA have also been studied. The average half-life of mRNA measured in mouse morulae is 8 to 11 hours, while that in blastocysts is 14 to 26 hours (Kidder and Pedersen, 1982). As in HeLa cells (Singer and Penman, 1973) and rabbit blastocysts (Schultz, 1974), the mRNA decay profile in mouse blastocytes also has been observed to be biphasic with short-lived (about 6 hours) and long-lived (24 hours or more) components (Kidder and Pedersen, 1982). Such decay curves, however, probably represent an average of a continuum of decay of different classes of mRNA with different half-lives. The mixture of short-lived and long-lived components is consistent with studies on the continued synthesis and disappearance of certain polypeptides when morulae are cultured to blastocyst stages in the presence of the transcriptional inhibitor, α-amanitin (Braude, 1979a, b).

One group of mRNAs that has been studied in detail in the early mouse embryo is the set of transcripts derived from histone genes. The histone genes of mammals are part of a small multi-gene family with 10 to 20 different genes for each histone protein. Four H3 genes, three H2b genes and two H2a genes have been isolated from three separate mouse genomic clones (Sittman *et al.,* 1983; Graves *et al.,* 1985b). Two of the gene clusters are localized on chromosome 13, and the third is on chromosome 3. An S1 nuclease mapping technique has been developed to measure expression of the individual genes (Graves *et al.,* 1985b). When this technique is applied to maternal mRNA derived from unfertilized mouse eggs and zygote-genome mRNA derived from the blastocyst stage, a number of changes are observed. A large amount (40 to 50%) of the histone H3 mRNA in the egg is complementary to the H3 gene located on chromosome 3 (H3.614) whereas only 14% of the histone H3 mRNA in the blastocyst is derived from the H3.614 gene (Graves *et al.,* 1985a). Similarly, nearly all the H2a mRNA in the egg is derived from the H2a.614 gene on chromosome 3, whereas only 30% of the H2a mRNA in blastocysts is complementary to the H2a.614 sequence. These and other data demonstrate that the same set of histone genes seem to be expressed in eggs and early embryos, but there are large differences in the relative abundance of certain histone mRNA types.

Changes in histone gene sets expressed during sea urchin development are also well-documented (Newrock *et al.,* 1978; Maxson *et al.,* 1983), but just as in the mouse embryo, the significance of these changes with respect to control of gene expression is not known.

PATTERNS OF PROTEIN SYNTHESIS IN THE EARLY EMBRYO

The ovulated mouse egg contains the machinery to synthesize proteins due to accumulation of rRNA, mRNA, tRNA, and ribosomes during oogenesis (Bachvarova, 1985). However, many of the ribosomes in the egg may not be functional when judged by their ability to form initiation complexes *in vitro* with a synthetic messenger RNA (Bachvarova and De Leon, 1977). Indeed, spare translational capacity of the fertilized mouse egg for injected globin mRNA is extremely limited (Ebert and Brinster, 1983). During the first 24 hours of post-fertilization development, there is little change in net protein synthetic rate, protein turnover, or total protein content (Brinster *et al.,* 1976; Merz *et al.,* 1981). During this same interval, there is a loss of 70 to 90% of the mRNA (Piko and Clegg, 1982; Giebelhaus *et al.,* 1983). It follows that much of the mRNA in the unfertilized egg is in a form where it is not used for translation or is used at a very low efficiency.

From a qualitative point of view, the changes in the pattern of protein synthesis during the early cleavage period are very marked. In a previous section we have already documented that many of the changes in the 1-cell to 2-cell transition period are post-transcriptionally controlled. It is important to note that some of the changes in protein pattern and cytoplasmic structure (*e.g.,* mitochondrial translocation; van Blerkom and Runner, 1984) that occur up to the stage of pronuclear fusion appear to be controlled by post-translational processes such as glycosylation and phosphorylation rather than translation of stored maternal mRNA (van Blerkom, 1981, 1985; Howlett and Bolton, 1985). Other changes up to the 2-cell stage do seem to depend on sequential activation of selected mRNAs by some translational control mechanism (Braude *et al.,* 1979; Cascio and Wassarman, 1982). In summary, there are fertilization-independent, fertilization-accelerated, and fertilization-dependent changes in polypeptide synthesis during the first cleavage. Some are due to post-translational modification, some are due to differential mRNA activation and others are due to differential polypeptide turnover (Howlett and Bolton, 1985). All of the latter mechanisms are involved in producing the well-described series of changes in polypeptide synthetic pattern that occur within a protein complex with a molecular mass of about 35,000 during the first 24 hours after fertilization (Levinson *et al.,* 1978; Braude *et al.,* 1979; Cullen *et al.,* 1980; van Blerkom, 1981; Flach *et al.,* 1982; Howlett and Bolton, 1985).

Changes in patterns of protein synthesis following fertilization or egg activation also occur in other systems. In the sea urchin embryo, there is a large increase in protein synthetic rate at fertilization (Grainger *et al.,* 1979) with accompanying changes in the qualitative pattern of protein synthesis (Evans *et al.,* 1983). For example, maternal mRNAs which code for four proteins (whose synthesis is barely detectable in the unfertilized egg) become actively translated to yield abundant products of synthesis after activation. Some of these proteins (termed cyclins) are destroyed every time the cell divides (Evans *et al.,* 1983). In addition, mRNAs for early histone variants are stored in the female pronucleus (Venezky *et al.,* 1981) but are not translated until after the first cleavage (Wells *et al.,* 1981). With continued development, a switch to late embryonic histone variants occurs (Maxson *et al.,* 1983). In the surf clam, *Spisula solidissima,* there is only a small increase in protein synthetic rate upon fertilization but a major change in the classes of proteins that are synthesized due to selective activation of maternal mRNAs also occurs (Rosenthal *et al.,* 1980; Tansey and Ruderman, 1983). Perhaps these changes are so dramatic because the *Spisula* egg has not yet undergone meiotic maturation. In any regard, striking examples of translational control of changing patterns of protein synthesis are documented in these studies.

An interesting set of polypeptides which appears at the early 2-cell stage of mouse development is a complex with approximate molecular weight of 67,000 to 70,000. Synthesis of these polypeptides is dependent upon new transcription since they do not appear in fertilized eggs cultured to the 2-cell stage in the presence of α-amanitin (Flach *et al.,* 1982; Bensaude *et al.,* 1983; Bolton *et al.,* 1984). One-dimensional peptide maps of the 68,000 (68K) and 70,000 (70K) components are not distinguishable from two heat shock proteins, hsp 68 and hsp 70 derived from cultured mouse F9 cells (Bensaude *et al.,* 1983). The developmental regulation of such heat shock or stress proteins is not unique to 2-cell mouse embryos. For example, hsp 70 mRNA accumulates in *Xenopus* oocytes (Bienz and Gurdon, 1982) but is translated in the oocyte only after heat shock treatment. After fertilization, translation of the hsp 70 mRNA cannot be induced by hyperthermia and cleavage stage embryos lack measurable hsp 70 mRNA and have no detectable hsp 70 synthesis (Bienz, 1984; Heikkila *et al.,* 1985). A number of hsp mRNAs also accumulate in *Drosophila* oocytes (Zimmerman *et al.,* 1983) and larval and pupal stages (Cheney and Shearn, 1983). It is interesting to note, however, that in spite of the developmental regulation of the hsp 68-70 genes, the cleaving mouse embryo is refractory to induction of additional hsp synthesis by exogenous stress-inducing stimuli. A heat-shock "incompetent" period, in which hsp 68-70 synthesis is not induced in response to environmental stress, has also been observed prior to the blastoderm stage of *Drosophila* embryos (Dura, 1981), the blastula stage in sea urchin embryos (Roccheri *et al.,* 1981), and the mid-blastula stage in *Xenopus* embryos (Bienz, 1984; Heikkila *et al.,* 1985). The ability to respond to stress by the synthesis of hsp 68-70 is acquired by the blastocyst stage in both the mouse (Wittig *et al.,* 1983; Morange *et al.,* 1984) and the rabbit embryo (Heikkila and Schultz, 1984). Failure of induction during cleavage may be related to the presence of constitutive levels of hsp 70 protein in the early embryos (Morange *et al.,* 1984) although the differential response to heat shock in the preimplantation embryo remains unexplained and awaits further study.

Although the patterns of protein synthesis during the first cell division have been shown to be derived from the translation of both maternal and zygote-genome derived templates, there is no evidence for the translation of oogenetic mRNA in the mouse embryo after the 4-cell stage. Processes such as compaction, cavitation, and blastocyst formation require transcription from the embryonic genome, although an element of post-transcriptional regulation is also involved (Kidder and McLachlin, 1985). For example, the transcription of templates necessary for the critical events in compaction of the 72-hour post-HCG 8-cell embryo are completed by the 4-cell stage (Kidder and McLachlin, 1985). Conversely, in the process of cavitation and development of the morula to the blastocyst, transcriptional and translational processes are tightly coupled (Braude, 1979a, b; Kidder and McLachlin, 1985).

From a qualitative point of view, two-dimensional gel patterns of proteins synthesized by 72-hour post-HCG morulae have been compared to those from embryos cultured *in vitro* to the blastocyst stage and to patterns from embryos arrested by culture in the presence of α-amanitin (Braude, 1979a, b). The major feature of these patterns is that the majority of the polypeptides show little change between the morula and blastocyst stages and are translated from messenger RNAs of relatively high stability, since they continue to be synthesized 24 hours later despite the presence of a transcriptional inhibitor. A small set of polypeptides (Braude, 1979b) fails to appear or to increase in intensity in the presence of the inhibitor and a similar number persists in the presence of the inhibitor when normally synthesis would have ceased prior to the blastocyst stage. The normal events ultimately do lead to the appearance of a number of specific polypeptides in both the trophectoderm and ICM cell lineages at the blastocyst stage (van Blerkom *et al.,* 1976; Handyside and Johnson, 1978; Brulet *et al.,* 1980; Howe *et al.,* 1980).

Quantitatively, the rate of protein synthesis remains at a relatively low level from fertilization to the 8-cell stage. Once the 8-cell stage is reached, there is a progressive increase in synthetic rate accompanying the transition of the morula to the blastocyst (Epstein and Smith, 1973; van Blerkom and Brockway, 1975; Brinster *et al.,* 1976; Abreu and Brinster, 1978). Ribosome numbers increase progressively with developmental stage due to active synthesis of rRNA and ribosomal proteins (La Marca and Wassarman, 1979). These rates of synthesis are sufficient for the production of about 2.5×10^6 ribosomes/embryo/h and can account for both the increase in ribosomal content and increased protein synthetic rate in the blastocyst (Piko and Clegg, 1982). An additional component may involve a shift in the pool of poly(A)+ RNA in the subribosomal fraction of morulae to the polysome fraction in blastocysts (Kidder and Conlon, 1985).

DNA METHYLATION IN EARLY DEVELOPMENT

Considerable interest has surrounded the field of DNA methylation in cell differentiation, because methylation of DNA sequences, for at least some genes in eukaryotes, is associated with inhibition of transcription (see Ehrlich and Wang, 1981, and Doerfler, 1983 for reviews). Five (5)-methyl cytosine appears as a minor base in the DNA of many organisms. It is produced by enzymatic (methylase) addition to some of the cytosine residues that are adjacent to guanine (m^5CpG) in genomic DNA. During DNA replication, the cytosine of the newly synthesized strand is usually symmetrically methylated as soon as a sequence is made. If a CpG pattern is not methylated, it will remain that way (Ehrlich and Wang, 1981). An alteration of the methylation pattern of a cell (hyper- or under-methylation) relative to a parental cell line can potentially lead to a heritable change that will be passed on from one cell generation to the next, generating a distinct cell lineage at the DNA level.

In mammalian cells, initial studies indicated that genes were undermethylated in tissues in which they were expressed during development or cell differentiation and hypermethylated when inactive. This appears to be true for about one-third of the thirty genes analyzed to date (Kolata, 1985). In addition, many of the so-called "housekeeping genes," which are active in all cell types are undermethylated at their 5′-terminal initiation sequences. In another 20% of the genes, there is no correlation between methylation and gene activity (Kolata, 1985). Since there is a lesser degree of methylation of cytosine residues in lower vertebrates and essentially no methylation in *Drosophila* DNA (Ehrlich and Wang, 1981), association of methylation with gene expression may be restricted to mammalian cells. In addition, methylation may be associated only in a secondary way with respect to expression of genes rather than being the primary event in turning off genes. Nonetheless, interesting patterns of DNA methylation are associated with formation of the first distinct cell lineages in the mouse embryo.

At about 4.5 days of development, just before implantation of the blastocyst, ICM cells become either primitive ectoderm or primitive endoderm. Along with the trophectoderm layer, the primitive endoderm gives rise to extraembryonic structures while the primitive ectoderm contributes to the three germ layers of the embryo (Papaioannou, 1982; Gardner, 1982). On the basis of methyl-sensitive restriction endonuclease digestion patterns of mouse satellite DNA and dispersed repetitive sequence, it has been shown that these sequences are undermethylated in all derivatives of the extraembryonic lineages compared to those in primitive ectoderm or DNA of adult tissues (Chapman *et al.,* 1984). Similarly, the DNA of the trophoblast component of rabbit blastocysts has been shown to be undermethylated (Manes and Menzel, 1981). Recently it also has been demonstrated that the undermethylation of DNA in extraembryonic structures of the mouse embryo is not restricted to repetitive sequences and includes alpha-fetoprotein, albumin, and major urinary protein structural gene sequences (Rossant *et al.,* 1986). The same sequences are heavily methylated in embryonic tissues as early as 7.5 days of development (Rossant *et al.,* 1986). These findings along with other studies (Razin *et al.,* 1984; Young and Tilghman, 1984) confirm that major differences in DNA methylation occur as cell lineages are established in the early embryo.

In the process of establishing different methylation patterns in the early cell lineages, both *de novo* methylation and demethylation probably occur. In general, sperm DNA is highly methylated with respect to structural genes, although satellite DNA sequences are less methylated in sperm than in adult tissues (Waalwijk and Flavell, 1978; Sanford *et al.,* 1985). Oocyte DNA is undermethylated with respect to repetitive DNA sequences (Sanford *et al.,* 1985). Retroviral sequences introduced into the preimplantation embryo become *de novo* methylated, but sequences introduced into post-implantation stages (8-day mouse embryos) escape methylation (Jahner *et al.,* 1982). Taken together with the fact that the DNA of cleavage-stage rabbit (Manes and Menzel, 1981) and mouse embryos (Singer *et al.,* 1979) has levels of methylation similar to that of adult tissues, it is possible that the *de novo* methylation of sperm and egg DNA may occur early. If this is so, it follows that the differentiating trophectoderm and primitive endoderm lineages must undergo extensive demethylation processes. There are insufficient data to make firm conclusions about the timing of *de novo* methylation events in the embryo, and the possibility that *de novo* methylation does not occur until the primitive ectoderm lineage is established must also be entertained (Sanford *et al.,* 1985). Although it is clear that the methylation pattern of the alternate lineages is distinct, the significance of this observation with respect to subsequent differentiation and gene expression in each line remains to be established through further research.

CONCLUDING REMARKS

Analysis of the molecular biology of the early mouse embryo is difficult because of its small size and limited numbers of available embryos. Nonetheless, the development of recombinant DNA techniques of high sensitivity recently has allowed approaches to problems of gene expression in early mouse embryos that previously could be studied only in systems where embryological material was more abundant. Using S1 mapping techniques, it has been possible to measure histone mRNA transcripts in mouse blastocysts that are present in as few as 600 copies per cell (Graves *et al.,* 1985a). *In situ* hybridization methods have been developed for sea urchin embryos that can detect mRNAs in defined cell types in as few as 50 copies or less per cell (Cox *et al.,* 1984; Angerer and Davidson, 1984). This molecular cytological approach can be applied to a small number of embryos and is potentially well-suited to investigation of early mammalian embryos. Although laborious to construct, cDNA libraries from mRNA from mouse oocytes and blastocyts have now been made (McConnell and Watson, 1986). In time these libraries will aid the identification of genes expressed at particular stages of early development.

A key feature of development in the mouse that requires future emphasis is the identification of marker genes that are activated in time and space during the period of early implantation, germ line formation in the embryo proper, and early morphogenesis and organogenesis. The techniques now exist to study patterns of gene expression during this critical phase. I purposely have not included extensive discussions of the use of genetically engineered transgenic mice in this review because, to date, this approach has yielded information primarily on tissue specificity of gene expression in fetal or adult organs. Nonetheless, expression from fusion plasmids microinjected into mouse pronuclear zygotes has also been observed in cultured preimplantation embryos (Brinster *et al.,* 1982; Pedersen and Meneses, 1985; Chen *et al.,* 1986). These approaches are applicable to gene expression studies during early development and during cell lineage formation and offer exciting prospects for elucidation of mechanisms underlying cellular commitment events in the embryo.

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MIGRATION OF *LIMULUS* FOR MATING: RELATION TO LUNAR PHASE, TIDE HEIGHT, AND SUNLIGHT

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ABSTRACT

In the spring, horseshoe crabs (*Limulus polyphemus*) along the eastern coast of North America migrate toward shore to build nests close to the water's edge. In 1984 the mating season near Woods Hole, Massachusetts, extended from 14 May to 7 July. Mating activity during this period fluctuated with the phase of the moon, the height of the tide, and diurnal changes in daylight. As the moon approached new and full phases, large numbers of animals migrated into the intertidal zone to mate and build nests. They appeared 1–2 h before high tide, and returned to deep water about 2 h after high tide. No mating activity occurred during low tides. The two daily high tides in this region are unequal in height. The inequality is greatest during new and full moons. At these times most animals migrated toward shore on the higher tide, which occurred in the late afternoon and throughout the night. As the moon passed through quadrature the tidal inequality diminished and reversed. Mating activity changed accordingly: shore migration diminished, becoming nearly equal on the two equally high tides, and in several days shifted to the higher high tide. The cue for shifting appears to be the first light of dawn and not the height of the tide itself. We suggest that the migration of *Limulus* involves several sensory modalities including vision.

INTRODUCTION

Much has been learned about vision from research on the horseshoe crab, *Limulus polyphemus.* The classic studies of the *Limulus* lateral eye by H. K. Hartline and his colleagues revealed basic mechanisms of retinal function common to many animals (Hartline, 1969; Ratliff, 1974). More recent studies show that the *Limulus* visual system is an excellent example of the central modulation of retinal sensitivity (Barlow *et al.,* 1977; Barlow, 1983; Barlow *et al.,* 1985). Efferent nerve signals generated by a circadian clock located in the brain increase the sensitivity of the lateral eyes at night. Laboratory studies show that the circadian increases in retinal sensitivity can be detected behaviorally (Powers and Barlow, 1985), and field studies indicate that vision mediates some aspects of mating behavior (Barlow *et al.,* 1982). Further studies of the visual component of *Limulus* mating behavior should enhance our understanding of the functional organization of the visual system (Barlow *et al.,* 1987). As a first step we carried out a detailed investigation of mating activity itself.

Limulus mates in intertidal zones along the eastern coast of North America and along much of the coast surrounding the Gulf of Mexico (Shuster, 1979). These coastal regions are dynamic environments. Their physical characteristics undergo large, periodic changes due to movements of the earth and moon. Although such

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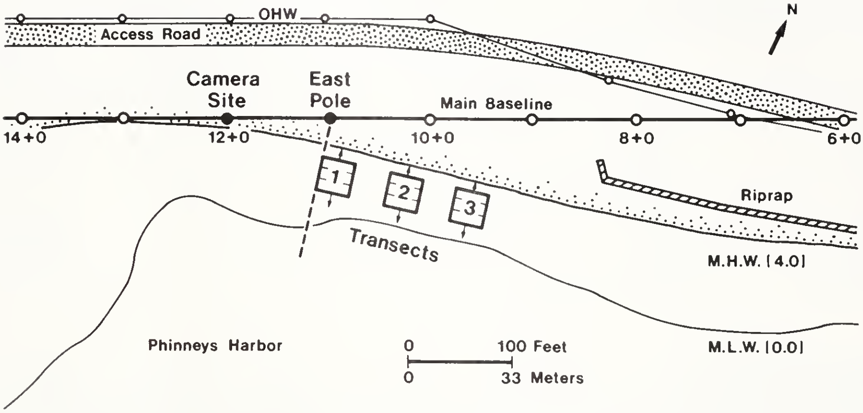


Mashnee Dike Research Site

FIGURE 1. Map of the research site on Mashnee Dike. We defined the East Pole as the steel pipe located in 1933 by the U. S. Army Corps of Engineers at 70°37′46″W and 41°46′34″N. Other pipes placed at 100 ft intervals mark the main baseline. All counts were taken in transects 1, 2, and 3 as defined by the shoreline and the East Pole of the main baseline. Broken lines indicate sectors of the transects. The water elevation at mean high water (M.H.W.) is 4.0 feet above mean low water (M.L.W.). The access road on the dike is lined with utility poles that support overhead wires (OHW). The camera site 100 ft from the East Pole was used in a concurrent study of visually guided behavior of *Limulus* (Barlow *et al.*, 1984, 1986).

dramatic fluctuations would appear to produce a forbidding environment, many marine and terrestrial species either inhabit the intertidal zones permanently or migrate into them for important biological activities (review: Neumann, 1981). Many such animals synchronize their activities with the major environmental rhythms of the intertidal zone. Preliminary observations indicated that lunar phase, tide height, and sunlight influence the mating activity of *Limulus* (Howard *et al.*, 1984).

We report here a quantitative study of the imigration of *Limulus* to the tidal zone for mating. We examine the temporal relationships between migratory behavior and the seasonal, lunar, tidal, and daily light cycles.

MATERIALS AND METHODS

We quantified mating activity as the number of animals appearing within the intertidal zone. This definition is based on prior observations that large numbers of adult horseshoe crabs regularly appear along the coastline in conjunction with breeding activities (Schuster, 1957, 1958, 1979; Cavanaugh, 1975; Rudloe and Herrnkind, 1976; Rudloe, 1978, 1979, 1980; Barlow *et al.*, 1982, 1984; Cohen and Brockmann, 1983; Howard *et al.*, 1984). The data were gathered between 13 May and 12 July 1984. Observations were also made throughout the 1985 and 1986 mating seasons.

Research site

Figure 1 shows the location of the research site at Mashnee Dike, Cape Cod, Massachusetts. Mashnee Dike is a narrow strip of sandy beach constructed by the U. S. Army Corps of Engineers in 1933 to connect Mashnee Island with Cape Cod. Permanent landmarks from the original survey include iron pipes that define the main baseline of the survey. One of the pipes, designated the "East Pole," stands about 2 ft. high at longitude 70°37′46″ West and latitude 41°46′34″ North. No houses or artificial lights are located within 0.8 km of the research site.

TABLE I

Number of Limulus counted in 1984

	Pairs		Single		
	Moving	Nesting	Male	Female	Total animals
Near	601	1378	2866	2	6826
Middle	1000	550	2621	1	5722
Far	614	129	958	3	2447
Females	2215	2057	—	6	4,278 (29%)
Males	2215	2057	6445	—	10,717 (71%)
Total animals	4430	4114	6445	6	14,995

Counting procedures

To sample animal numbers in the area, we established three 10 m × 10 m transects along the shoreline, using the East Pole and the water's edge as reference points. This technique is similar to one used by C. M. Cavanaugh (pers. comm.). The west side of Transect 1 (see Fig. 1) was defined by a line perpendicular to the shoreline and passing through the East Pole. The north side of all three transects was defined by the water's edge. The transects were separated from each other by 10 m. Two reflective posts were driven into the sand several meters above mean high water (M.H.W.) along the continuation of the east and west borders of each transect. This allowed us to determine their boundaries for each counting session. The position of the transects thus remained fixed in the longshore direction, shifting along the onshore-offshore direction as the tide flooded and ebbed (Fig. 1).

Most counts were taken around the times of the two daily high tides because previous observations indicated that few animals appeared at this site during low tides (Barlow *et al.,* 1982; Howard *et al.,* 1984). On three occasions we counted animals every half hour around the clock, to confirm that *Limulus* appeared in the intertidal zone only during high tides. At all other times counts were made every half hour for a period of up to 3.5 h beginning about 1 h before predicted high tide.

In each counting session the observer began at the west side of Transect 1 and waded toward the east side counting the animals within the "near" sector, the third of the transect adjacent to the water's edge. Upon reaching the east side, determined by lining up the reflective posts on shore, the observer moved offshore about 4 m, waded toward the west side, and counted the animals in the "middle" sector (the middle third of the transect). The observer then moved to about 8 m offshore, walked across the transect, and counted the animals in the "far" sector (the outer third of the transect; depth < 1 m). This procedure was repeated for Transects 2 and 3 with the entire session requiring about 10 min.

Animals were categorized as they were counted, and the number in each category was noted for the three sectors of each transect. The categories were "moving pairs" (female and male in amplexus and freely swimming), "nesting pairs" (female-male pair had burrowed into the sand), and unpaired "single males." Frequently we noted how many single males were "freely swimming" and how many were associated with a nest ("satellite males"). With this counting technique, no animals were overlooked in the transects during the day. We estimate that less than 5% were overlooked at night. The animals move slowly enough that counting the same animal twice would seldom occur.

Because unpaired females are rarely found on mating beaches (Cavanaugh, 1975;

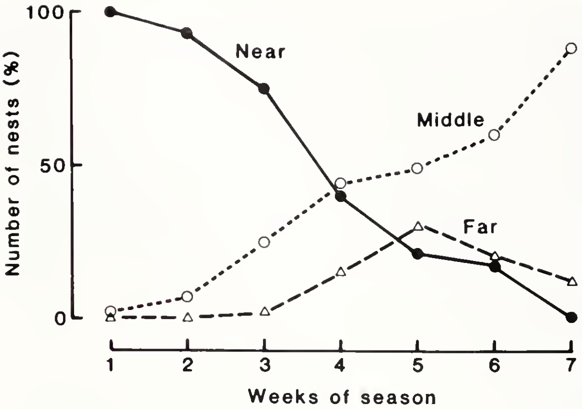


FIGURE 2. The relative number of nests counted in three regions of the transects varied across the mating season. The three symbols give the distribution of nests in the sectors as percentages of the total number of nests counted during a given week. In the early part of the season most animals nested in the near sector of the transects, closest to the water's edge. By the end of the season most animals were nesting in the middle sector. Few nests were ever observed in the far sector of the transects. Sectors are indicated in Figure 1.

R. B. Barlow, Jr., unpub. obs.), we did not examine every free-swimming animal. However when we suspected that a free-swimming animal might be female because of its large size, we picked it up to identify its gender. Only six were females. In several counting sessions in 1985 and 1986 we identified the gender of all unpaired animals and each time found about 0.5% were females.

Tide measurements

To estimate the times of high tides in planning our counting sessions we used the *Cape Cod Canal 1984 Tide Tables,* published by the New England division of the

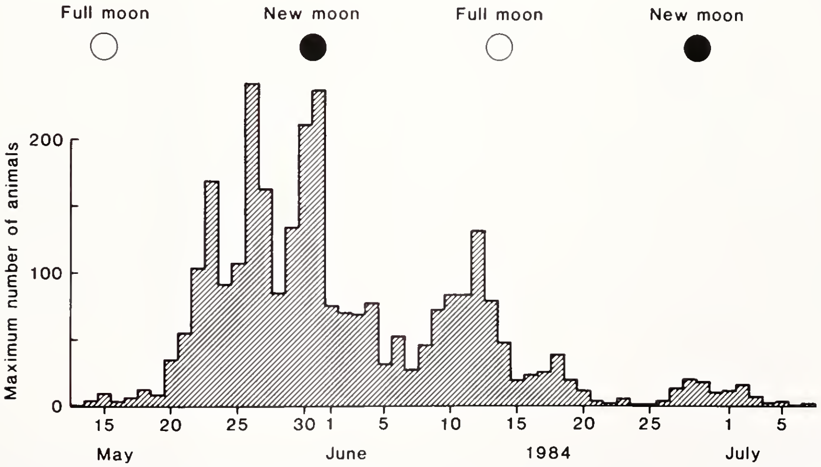


FIGURE 3. Maximum number of *Limulus* counted in the transects each day of the 1984 mating season at Mashnee Dike. The height of each bar shows the largest total number of males and females recorded in a single half-hourly counting session during the solar day. The open and filled circles indicate the full and new moons of 14 and 30 May and 13 and 28 June (Eldridge, 1984).

U. S. Army Corps of Engineers (1983). The most appropriate tables were those for Wings Neck, located 0.6 nautical miles southwest of Mashnee Dike. Except during foul weather, the predicted time of high tide at Wings Neck differed from that at Mashnee Dike by less than 5 min. The actual time of high tide at Mashnee Dike was determined by the observer during each counting session by noting the distance from the East Pole to the water's edge.

Tide height was measured by the U. S. Army Corps of Engineers with a nitrogen gas gauge submerged in the approach channel to the New Bedford-Fairhaven Harbor. Although the approach channel is located 11.6 nautical mi. from our observation site, the tide heights measured in the channel are within 1–2 cm of those at Mashnee Dike (F. Morris, pers. comm.).

RESULTS

Mating season

The mating season of *Limulus polyphemus* at Mashnee Dike in 1984 lasted 55 days, from 14 May to 7 July (Fig. 3). Table I gives the numbers of males, females, and pairs counted in the transects during this period. Because we did not tag individual animals, the values provide information on the relative proportions of animals in the different categories but not their absolute numbers. Absolute values would be influenced by animals that remained in a transect for more than one counting period, moved from one transect to another, or returned to the mating area on subsequent days.

When all transects are considered together the numbers of moving and nesting pairs were approximately equal (2215 and 2057, respectively), and were less than the total number of single males (6445). If each pair is counted as 1 male and 1 female, more than twice as many males (10,717) as females (4,278) were sighted in the transects. For the sessions during which we divided single males into free-swimming and satellite, their ratio was approximately 80:20. As anticipated, very few single females were seen.

Limulus were not uniformly distributed across all three sectors of the transects (Table I). More moving pairs were counted in the middle sector than in either the near or far sectors. More nesting pairs were counted in the near sector, with progressively smaller numbers in the middle and far sectors. Single males were concentrated in the near and middle sectors, where the number of pairs was greatest.

The distribution of the nesting pairs within the transects changed systematically as the season progressed. Relatively more nests were found in the near sector along the water's edge in the beginning of the season, and in the middle sector later in the season (Fig. 2). Few were seen in the far sector. Nesting pairs were not observed in the transects until 19 May, five days after the first moving pairs appeared. Nests appeared first in the near sector (on 19 May), then in the middle sector (on 21 May), and finally in the far sector, sporadically after 26 May. The shift of nests away from the shoreline as the season progresses may reflect a change in predation or an influence of temperature.

Semilunar rhythm

Mating activity was correlated with phases of the moon (Fig. 3). Large numbers of animals were counted each day before and during the new moon on 30 May and around the full moon on 13 June. A smaller peak occurred near the new moon on 28 June. The total number of nests counted in the transects each solar day followed

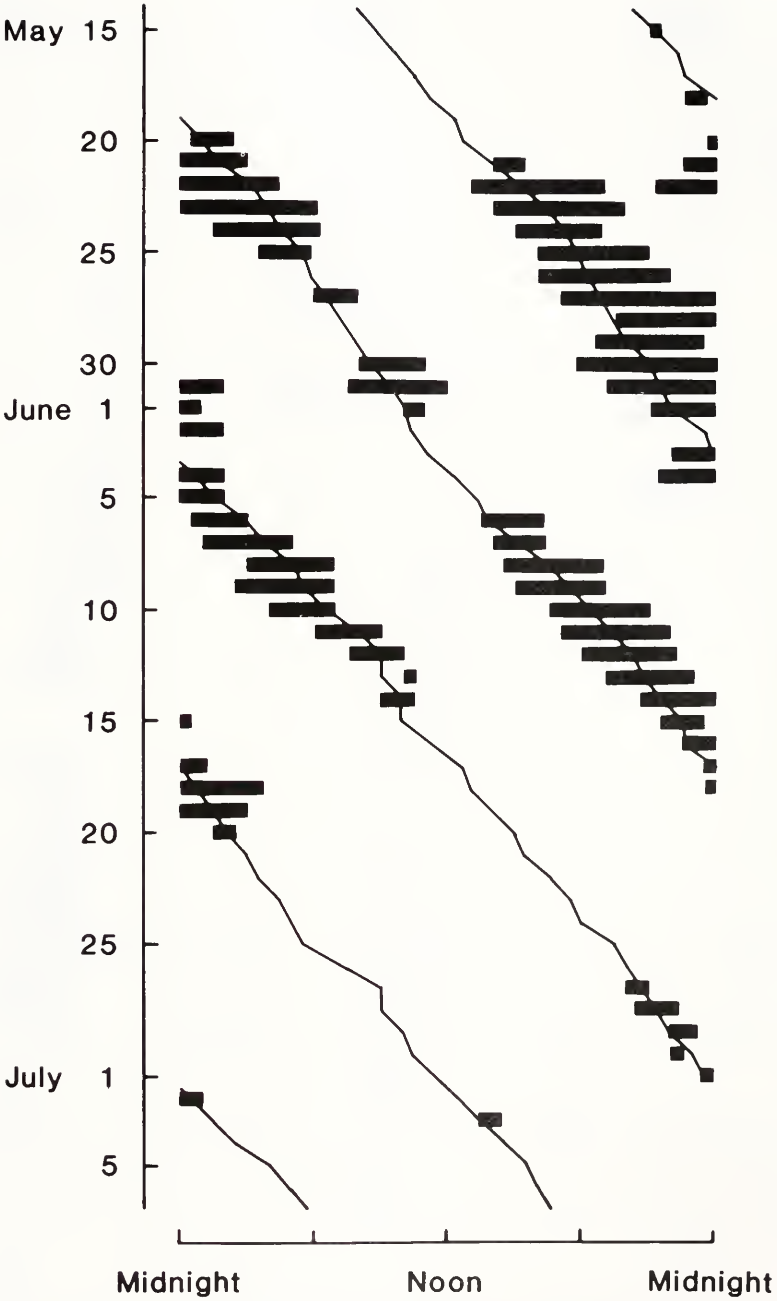
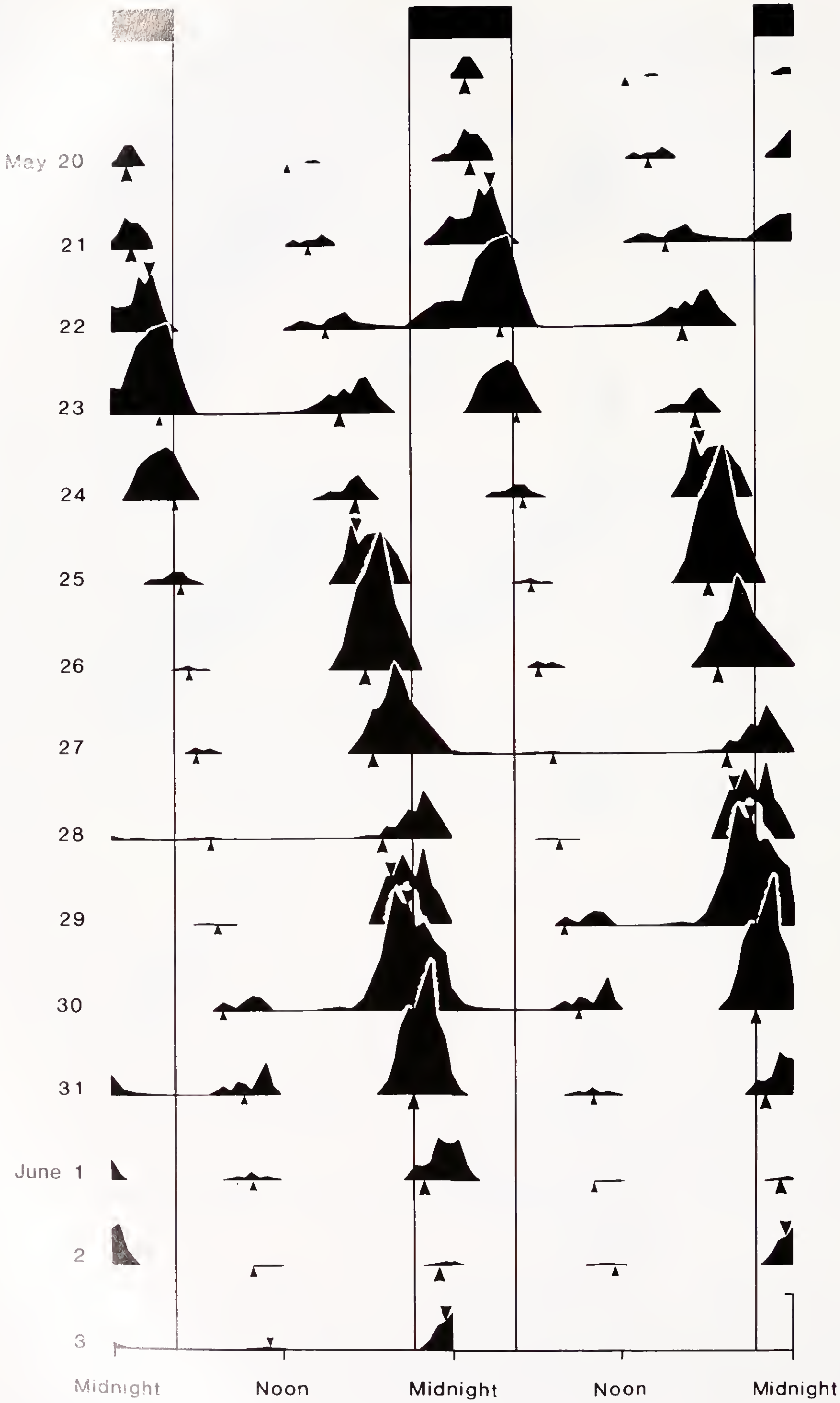


FIGURE 4. Daily mating activity at Mashnee Dike between 14 May and 7 July 1984. The bars indicate the times during which at least 10 *Limulus* were counted in the transects. Refer to Figure 5 for the duration of daily counting sessions during the peak of the season. The lines connect the times of each daily high tide as it advanced through the solar day. The lines have slopes of about 50 min day -1, indicating that each tide crests at intervals of 24.9 h, the length of the lunar day.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the same pattern. The fluctuations in migratory activity thus had a period of about 15 days, indicative of a semilunar rhythm.

A thorough quantitative study was not achieved in 1985 because many animals were removed from the area by fisherman during the height of the season. Nonetheless, the mating activity of the residual population exhibited pronounced fluctuations comparable to those in 1984. The first large peak of activity coincided with the new moon on 23 May and subsequent peaks occurred during the full moon on 3 June and the new moon on 17 June. Again the fluctuation in mating activity had a period of about 15 days and the season lasted for about 50 days.

Tidal rhythm

Mating activity occurred only during high tides (Fig. 4). The bars give the periods during each day when 10 or more animals were observed in the three transects. Mashnee Dike is subjected to a semidiurnal tidal regime, and the diagonal lines connect the actual times at which each of the two tides reached maximum height throughout the season. The close correlation between the lines and bars shows that significant numbers of animals appeared in the transects only at the high tides.

The tidal component in the daily pattern of mating activity persisted throughout the season. A line through the middle of the periods of activity (bars) associated with either high tide would have a slope of about 50 min per day. This equals the slope of the line representing the times of the high tides. Thus, daily episodes of mating activity advance through the solar day along with the high tides each with periods approximating 24.9 h, the length of the lunar day.

Figure 5 shows the distribution of animals during the half month of greatest mating activity (20 May to 3 June). Small vertical arrows indicate the times of high tides during this period. High tides occurred near midnight and noon on 20 May and progressed through the day to noon and midnight respectively by 3 June. Mating activity over this period occurred during both daily high tides with peak activity generally observed about 1–2 h after tidal crests. The animals then began leaving the nesting area to migrate offshore.

On 23, 28, and 30 May we counted animals in the transects every half hour for 30 hours or more. These long-term counts clearly show that no animals were present in the transects during low tides. In addition, underwater observations using SCUBA diving gear revealed very few moving and no buried *Limulus* within 100 m of the shoreline during low tide. Instead, numerous animals, both paired and single, were found on the bottom at depths up to 8 m between 100 and 400 m offshore. Some were swimming, some were stationary, but none were nesting.

More animals were observed during one of the two daily high tides than during the other. From 20 May to 24 May more appeared on the early morning tide than on the midday tide. These differences were large, approaching 200:1. On 24 May, the difference declined and on 25 May it reversed, with more *Limulus* appearing on the afternoon tide. On successive days, most animals continued to migrate to shore on

FIGURE 5. Daily distributions of horseshoe crabs in the transects between 20 May and 3 June 1984. Counts were taken only during the hours marked by baselines. For example, animals were counted around the clock on 22–23 May, 27–28 May, and 30–31 May and near the times of high tides (vertical arrows) on other days. Large arrows indicate the higher daily tides. The flanks of some distributions (about 10%) have been extrapolated when full counts were not available. Note that more animals appeared in the transects during the early morning tides from 19 May to 24 May and during the evening tides after 25 May. Dark bars and vertical lines indicate darkness (see Fig. 9). The data are double-plotted to emphasize the periodicity of the mating activity. Vertical scale equals 100 animals.

this tide as it progressed through the late afternoon and into the night. The animals abandoned the tide only after it moved past dawn on 7 June (not included in Fig. 5). They then shifted to the opposite (afternoon) tide and repeated the cycle. In the following sections we present evidence that these tidal preferences are related to the relative heights of the tides.

Semidiurnal inequality of tide height

On most days during the mating season one tide was clearly higher than the other. Such inequalities in tide height are caused at certain latitudes by the moon's monthly declination north and south of the plane of the ecliptic. This declination produces asymmetries in the system of tide-generating forces such that two tidal waves of different heights occur each day at the northern and southern latitudes whenever the moon is not over the equator (Wylie, 1979). During the spring the pattern of tidal inequality in the Cape Cod area produces maximal differences in the heights of successive tides twice monthly at full and new moons. These diminish over the following two weeks and reverse twice monthly during the first and last quadratures.

Figure 6 shows the actual (top) and predicted (bottom) tide heights at Mashnee Dike from 12 May to 3 June 1984. This period includes the full moon of 15 May, the new moon of 30 May, and the 15-day period covered in Figure 5. One of the two daily tides is indicated by a stippled bar, the other by a black bar. The predicted changes in tides follow a relatively smooth function, whereas the actual changes do not because they are influenced by unpredictable weather conditions. However, both exhibit maximal flood and ebb tides at the full and new moons, with neap tides at the lunar quadrature on 23 May. The daily low tides were nearly equal in height but the high tides were not. The inequality in high tides was greatest during the full moon and minimal near quadrature on 23 May when it reversed and became large again at new moon.

Mating activity on Mashnee Dike was closely related to the magnitude of the inequality between high tides. Figure 7 compares actual tide measurements with mating activity over the 8-day period from 20 to 27 May. On 20, 21, and 22 May, the daily high tides differed in height and more animals were counted within the transects during the higher tides, which occurred in the early morning hours before dawn. This was also the case on 25, 26, and 27 May when the majority of animals again appeared during the higher high tides, which occurred in the afternoon on those days. The animals' preference for the afternoon tide grew as the tidal inequality increased, and continued as this tide progressed through the evening and into the early morning hours.

Limulus did not, however, always prefer the higher high tide. Even though the tidal inequality diminished and reversed, the majority of animals continued to populate the early morning tides, which were slightly *lower* than the afternoon tides. They switched to the higher afternoon tide on 25 May, three days *after* tidal reversal. This behavior was repeated during the next tidal cycle, when they again switched to the afternoon high tide three days after reversal of the tidal inequality at the lunar quadrature on 6 June. We believe these exceptional days are significant for understanding factors that control *Limulus* mating activity, and we will consider them in more detail below.

Not only were more animals generally counted during the higher high tides, but the relative number appearing in association with any two consecutive high tides was roughly proportional to the relative difference in the heights of the two tides. This point is illustrated in Figure 8. In general, when the difference in the heights of the two high tides was large, as on 29 May, the degree of preference was also large. When

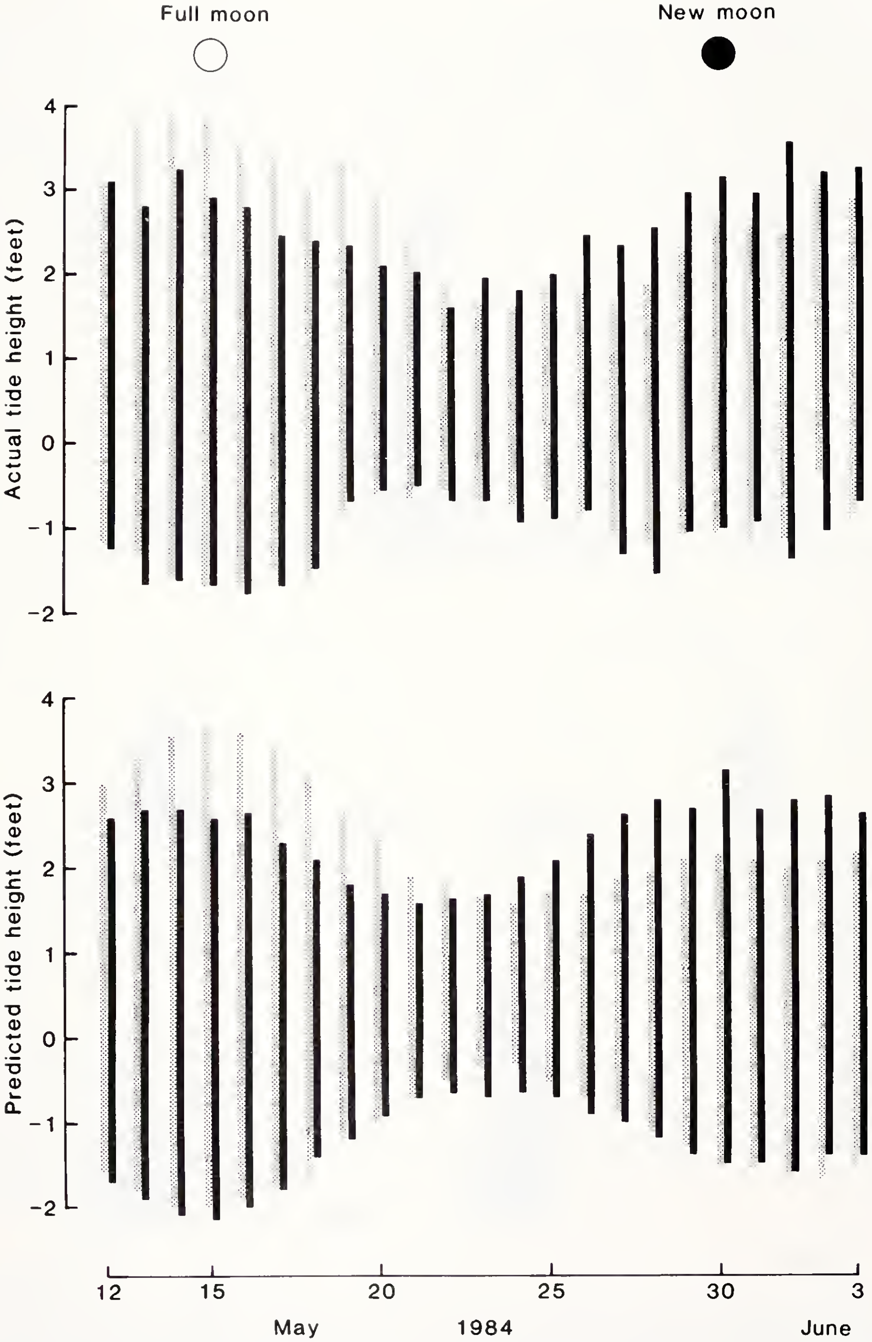

FIGURE 6. Predicted (bottom) and actual (top) heights of tides at Mashnee Dike during the first half of the 1984 mating season (Army Corps of Engineers). One of the two semidiurnal tides is indicated by black bars and the other by stippled bars. The tidal excursions were maximal during full and new moons with one tide (stippled bars) higher during the full moon and the other (black bars) during the new moon. Excursions were minimal during the lunar quadrature on 23 May when the inequality in heights reversed. Note that the heights of the low tides did not differ much over this period.

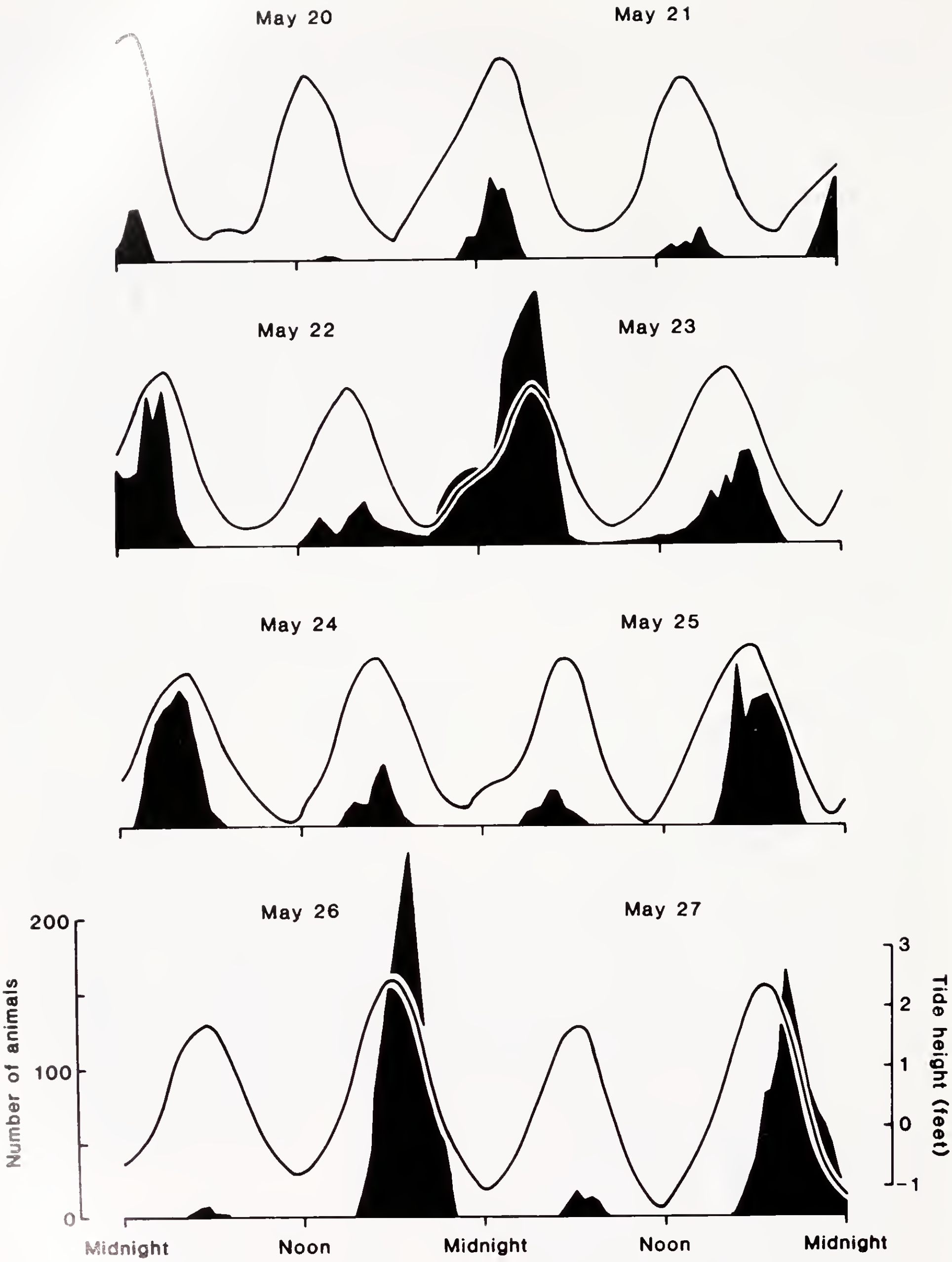

FIGURE 7. Comparison of tide height with the number of horseshoe crabs counted in the transects during the week of 20 to 27 May. From 20 to 22 May more animals appeared on the early morning tide, which was the higher of the two daily tides. On 23 May most animals remained with the early morning tide even though the inequality had reversed and the afternoon tide was higher. This behavior persisted until 25 May, when the animals' tide preference switched to the evening tide.

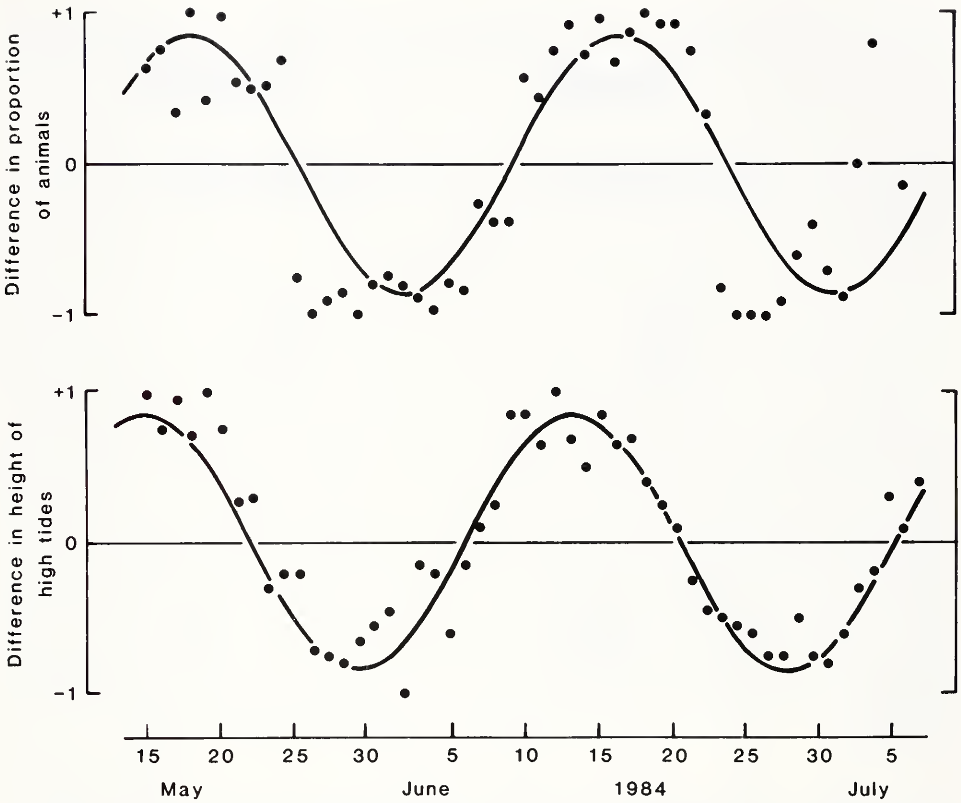

FIGURE 8. The relative proportion of animals observed on a given high tide (top) corresponds to the relative height of the tide (bottom). The difference in proportion of animals is equal to the difference between the total number of animals observed on two semidiurnal high tides divided by the sum observed on both tides. Each point on the bottom graph represents the difference in heights of the pair of semidiurnal high tides in feet. A sinusoid with a period of one lunar month (29.4 days) and an amplitude of 0.85 was fitted to the tidal data by eye. The same function shifted by 3.2 days is plotted in the top half of the figure.

the difference in tide height was small, as on 6 June, the degree of preference was small. But the correlation was not perfect. The sinusoidal function with a period of 29.4 days that describes the tidal data in the bottom half of Figure 8 must be shifted to the right by 3.2 days to fit the behavioral data. This shift implies a phase lag of nearly 3 days between changes in relative tide height and changes in the animals' preference for one tide over the other.

Sunlight

Mating activity was more strongly correlated with the tidal inequality than with light or darkness. For example, from 20 May to 3 June most animals (77%) migrated into the transects during the highest high tides indicated in Figure 5 by large arrows. These tides occurred from 1530 to 0330 h as shown by the stippled bars in Figure 9. Over this 15-day period half of the animals appeared in the transects during light (1530 to 2100 h) and half during darkness (2100 to 0330 h). We conclude that *Limulus* generally prefer to mate on the highest tides regardless of when they occur.

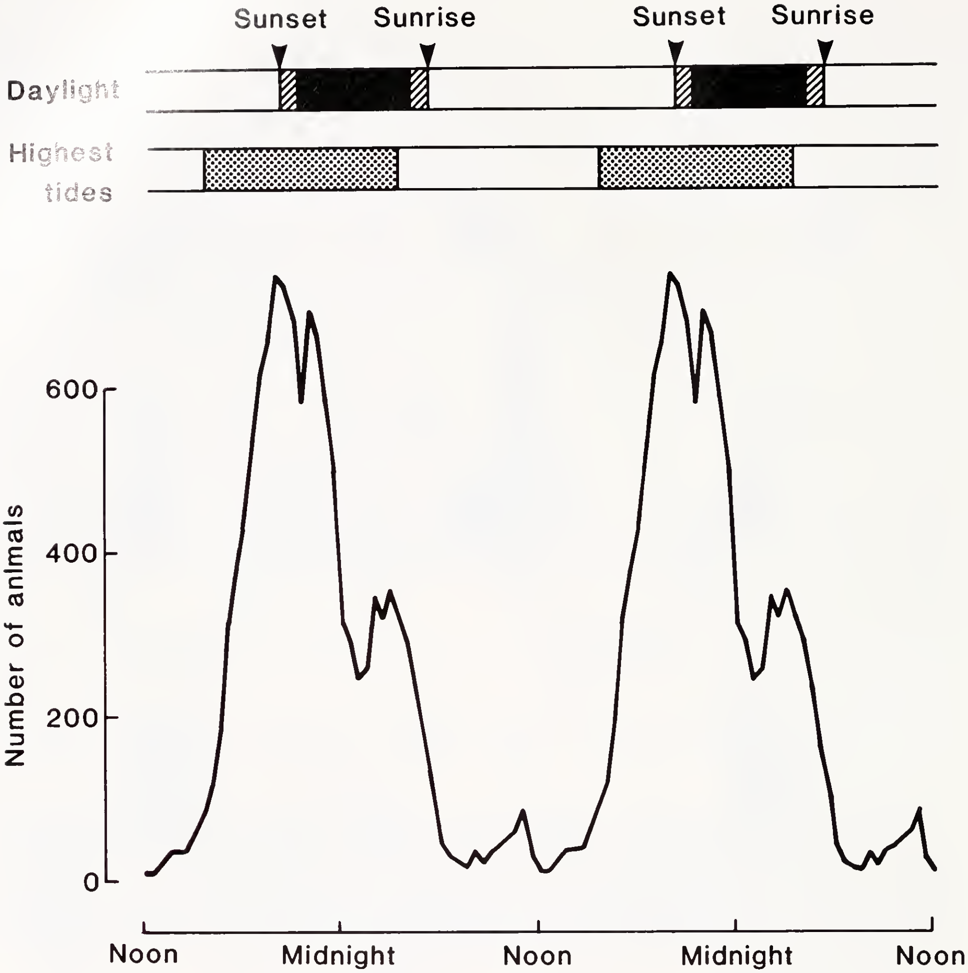

FIGURE 9. Comparison of mating activity with daylight, darkness, and the tidal inequality. The number of animals counted from 20 May to 3 June was plotted as a function of time of day by summing every half hour the counts shown in Figure 5. The data are double-plotted. The times of sunset (2010 h) and sunrise (0510 h) are given at the top with dusk (2110 h) and dawn (0410 h) indicated by cross hatching and darkness by black bars. Because these times changed slightly with each day the data are shown for 27 May, which is in the middle of the period. The stippled bars give the times of occurrence of the higher of the two daily tides over the 15-day period. Note that the peak number of animals was observed before sunset.

DISCUSSION

The mating activity of *Limulus polyphemus* is related to the environmental changes produced by periodic motions of the earth and moon. The yearly rotation of the earth about the sun produces seasonal changes in daylength and in ocean temperature. The daily rotation of the earth about its axis produces diurnal changes in daylight and semidiurnal changes in the levels of most oceans. The monthly rotation of the moon about the earth modulates the tidal flows of the oceans and produces periodic inequalities in the daily tides in the northern and southern hemispheres. All of these astronomical events affect the physical environment of intertidal zones, and this study indicates that all appear to influence *Limulus* mating behavior within the intertidal zones of Cape Cod, Massachusetts.

Time of year

Limulus mates seasonally. The animals entered and built nests in the intertidal zone at Mashnee Dike for about 8 weeks in 1984, beginning in mid-May and ending in early July (Fig. 3). This was also the case for the neighboring shores of Buzzards Bay, Narragansett Bay, Vineyard Sound, and Nantucket Sound (R. B. Barlow Jr., unpub. obs.). The time of the year and duration of the mating season in 1984 at Mashnee Dike was similar to that of other years from 1982 to 1986. The mating season is longer in the Gulf of Mexico and farther south along the eastern coast of North America, generally beginning earlier in the spring and ending later in the summer or even late fall (Schuster, 1979; Rudloe and Herrnkind, 1976).

The choice of spring for mating undoubtedly reflects periodic environmental factors caused by the earth's rotation about the sun. The most prominent factors are an increase in the duration of daily sunlight and a warming of the waters in the northern hemisphere. At more southern latitudes, the waters warm sooner and the days are longer in early spring. These differences may explain the earlier mating seasons in the southern latitudes if *Limulus* initiated mating when a particular ocean temperature and/or daylength was achieved. Either mechanism appears plausible. *Limulus* is a poikilotherm and its overall locomotor activity is influenced by ambient water temperature (R. B. Barlow, Jr., unpub. obs.). Also, the *Limulus* visual system possesses a circadian clock which maintains an accurate record of seasonal changes in photoperiod (Barlow, 1982).

Lunar phase

Mating activity of a variety of intertidal invertebrates is associated with the lunar syzygies (Klapow, 1972; Enright, 1975; Neumann, 1976; Christy, 1978; Zucker, 1978; Saigusa, 1981). *Limulus* appears to be no exception: mating activity was maximal during new and full moons throughout the season. In both 1984 and 1985, *Limulus* began migrating to the beach after the full moon, in early May. In 1984, mating activity increased significantly in the last week of May as new moon approached, decreased rapidly in early June after the new moon, and then peaked twice again at the full and new moons in June. No activity was detected during the full moon in July. The pattern of mating activity in 1985 was similar in every respect to that in 1984 except that the season began about 10 days earlier in the year. This shift coincides exactly with the earlier appearance of the new moon in 1985.

Two previous studies report that *Limulus* mating activity on the Gulf Coast of Florida was also coordinated with new and full moons. In contrast to our results, both studies found maximal mating activity associated with full moons. Rudloe (1980) counted *Limulus* appearing at Mashes Sand, Florida, between March and November, 1977. Mating apparently began with the new moon of 19 March. Peak numbers of animals were observed in association with high tides between early April and early June. Although daytime tides were monitored throughout, night tides were only monitored from 18 May. Rudloe reported maximal mating during high tides at full moons, with no breeding during neap tides. However, substantial mating also occurred during new moons, especially at the beginning of the season when overall numbers were largest. Cohen and Brockmann (1983) also observed more animals in association with the full moon at Seahorse Bay, Florida, between 12 July and 10 September 1980. The reasons for the differences between our observations and those of Rudloe (1980) and Cohen and Brockmann (1983) are not known.

In the nesting area of Mashnee Dike, unpaired males far outnumber paired males (Table I). This result is consistent with observations in Florida (Rudloe, 1980; Cohen and Brockmann, 1983). As these authors also noted, many males mill about a nesting pair apparently attempting to dislodge the clasping male or to get close enough to deposit sperm where they might be effective. Although in our studies satellite males never dislodged a clasping male (see also Cohen and Brockmann, 1983), they did release sperm around the nesting pair and in the nest itself after the pair had left. Since fertilization is external, sperm release by satellite males may increase the possibility that their sperm will fertilize some eggs.

Tide height

Limulus migrated in great numbers into the intertidal zones during new and full moons, but only on high tides (Figs. 3, 4, 5). None migrated toward shore during low tides. Although significant numbers of animals mated on neap tides at the lunar quadrature, maximal activity was always observed during the highest high tides associated with the new and full moons. Numerous species synchronize their mating activity in the intertidal zone with the tides (for reviews see DeCoursey, 1976; Naylor, 1976; Neumann, 1981). For example, grunion bury fertilized eggs at the high-water mark during spring high tides and the larvae hatch about two weeks later when the next series of spring tides washes the upper beach (see Neumann, 1981). *Limulus* appears to have adapted the same strategy in Florida (Rudloe, 1980). In other areas along the East Coast of North America, however, *Limulus* nests subtidally in up to 1 m of water (see Cohen and Brockmann, 1983). Indeed, we observed nests being built in areas of the intertidal zone that are inundated by high tides twice a day every day during the lunar month (see Table I). The preferred region for nesting moved away from the high-water mark as the season progressed (Fig. 2). The location of nesting sites within the intertidal zone may be related to the relative risks of predation in different geographical regions and seasonal changes in such risks in a given region. In this regard, it is interesting that geographically separate populations of *Clunio,* a marine insect, appear to have adapted to the local conditions of the semimonthly tidal regime (see Neumann, 1976).

Semidiurnal inequality of tide height

Mating activity on Mashnee Dike was directly related to the tidal inequality (Fig. 8). Most *Limulus* individuals (77%) appeared in the transects on the higher high tides which occurred between 1530 and 0330 h (Figs. 5, 9). The greatest mating activity was observed on the highest spring tides in the early evening (1800 to 2400 h) during the new and full moons. In sum, tide height is a powerful predictor of mating activity at Mashnee Dike.

A study along the Gulf Coast of Florida also reported a positive correlation between the number of *Limulus* mating and the highest high tides (Cohen and Brockmann, 1983). However, another Florida study in a nearby area reported the opposite result: more mated on the lower high tides (Rudloe, 1980).

Cavanaugh (1975) reported that *Limulus* mated only after dark at Mashnee Dike. Our study does not support this conclusion. From 20 May to 3 June, half of the animals were counted in the transects during darkness (2100 to 0330 h), and half were counted during light. We conclude that *Limulus* generally favor the highest high tides whether they occur during the day or at night.

Coordination of behavior with the inequality of tide height is not unique to *Limulus.* In an elegant laboratory study, Enright (1972) showed that the endogenous locomotor activity of the intertidal isopod *Excirolana chiltoni* was directly correlated with the height of the daily high tides in its natural habitat along the shores of southern California. The region has a semidiurnal tidal regime of the mixed type, and the endogenous activity of the isopod in isolation was maximal during the higher high tide when the tidal inequality was also maximal. When Figures 1 and 2 of Enright's paper are replotted in the format of Figure 8 of the present paper, their similarity is striking: the ratio of *Excirolana* locomotor activity on consecutive high tides appears to be directly related to the ratio of tide heights. Activity decreased as the tidal inequality decreased, and increased again as the inequality increased. This circasemilunar rhythm is endogneous in *Excirolana,* but it may not be in *Limulus.* Under diurnal lighting *Limulus* occasionally exhibits nocturnal locomotor activity in the laboratory, and under constant darkness the locomotor activity sometimes exhibits an endogenous rhythm ($\tau \simeq 24$ h) for several days (Barlow and Palfai, 1971; J. Turnbull and R. B. Barlow, Jr., unpub. obs.). We have never observed an endogenous lunar or semilunar rhythm for *Limulus* under laboratory conditions.

How does *Limulus* detect the tidal inequality? A variety of cues are potentially available, particularly when the inequality is large. Indeed, the degree of preference for the highest tides was maximal around the times of maximal tidal inequality. However, as the tidal inequality diminished near lunar quadrature, more animals began to migrate to the intertidal zone during the lower high tides. This behavior would be understandable if the discrimination between the two high tides became progressively more difficult as their heights approached equality. The phase lag of 2–3 days between tidal inequality and animal inequality suggests that *Limulus* persisted in migrating to the beach on alternate high tides until some environmental factor signaled them to shift to the opposite high tide. As discussed below, we believe the signal to shift tides may be the first light of dawn.

Possible role of sunlight

Three aspects of our results suggest that *Limulus* may use only environmental cues to synchronize its shoreward migration with tidal inequalities. (1) As already noted, there is a 2–3 day phase lag between the reversal of the tidal inequality and the shift of animal preference to the opposite high tide (Fig. 7). (2) With few exceptions, the maximum number of animals observed in association with a high tide occurred *after* tide crest (Fig. 5). The exceptional tides were all close to dawn when the animals' preference for one high tide over the other was changing. To see this, compare the early morning distribution of 24 May with the afternoon distribution of 23 May in Figure 5. Note that near dawn the maximum number of animals occurred *before* tide crest. (3) On several occasions we observed a mass exodus of animals from the nesting area as the first light of dawn appeared on the horizon. The rapid movement of animals away from shore was quite unlike the normal decline of mating activity observed on ebbing tides at other times of the day. We suggest that the first light of dawn may serve as a cue for the animals to shift to the opposite high tide after it has become the higher tide.

The *Limulus* visual system is well equipped to detect the first light of dawn. In the early hours of the morning, the animals' visual sensitivity begins to undergo an endogenous transition from a highly sensitive nighttime state to a much less sensitive daytime state (Barlow, 1983). Although visual sensitivity is declining, it can still detect single photons (Kaplan and Barlow, 1976). Another important factor may be the massive turnover of photoreceptor membrane which is triggered at dawn by the first rays of light (Chamberlain and Barlow, 1979, 1984). Within minutes about 70% of the photosensitive rhabdom structure of every photoreceptor cell is torn down, leaving a small volume of rhodopsin-containing membrane to transduce light, and after one half hour the rhabdom structure is fully reassembled. Although the process of rhabdom renewal is analogous to the outer segment renewal of vertebrate rods and cones (Young, 1976), its function is not understood. However, such a massive metabolic event may disrupt vision, which males use to locate potential mates (Barlow *et al.*, 1982).

The change in migratory behavior near dawn may be triggered by a daily environmental event other than dawn. One possibility is the solar component of the tide, but an analysis of tidal components shows that the solar component does not peak near dawn at Mashnee Dike (R. Gregory-Allen, pers. comm.). The relationship between migratory behavior, dawn, and tide could be further tested by observing a population that nests in an area with a different tidal regime. Several such areas exist near Woods Hole but thus far we have not found one with a mating population of sufficient size.

Our data are consistent with the idea that the animals prefer the highest tides but are incapable of detecting the reversal of tide heights—perhaps because the difference in tide height is smallest when the reversal occurs. Because the tide reversal at Mashnee Dike always occurs before dawn (0300 h), we suggest that the animals stay with this early morning tide until it occurs after dawn, 2–3 days later. Then they shift their shoreward migration to the higher high tide. They continue to follow the afternoon high tide as it progresses through the night until it again occurs after dawn. Such a strategy does not require an endogenous rhythm of the sort detected in the isopod by Enright (1972). However, it does require information about the timing of the two flooding tides. An endogenous circatidal oscillator would provide this information, but studies with other animals indicate that exogenous factors such as water flow and bottom vibration alone are sufficient (Hastings, 1981; Neumann, 1978). It is also possible that *Limulus* possesses an hour-glass timing system which signals the animal at a fixed interval after a preceding event. Such hour-glass timing systems have been detected in other animals (Neumann, 1981). They do not require an endogenous oscillator.

Summary of Limulus *mating activity in 1984*

Figure 10 summarizes our findings during the 1984 mating season at Mashnee Dike. It shows the heights of the two high tides as "paths" through time. *Limulus* symbols indicate which high tide attracted the majority of animals on any given day.

At the beginning of the season we counted more animals during the higher tide that occurred in darkness. The animals continued to prefer that tide until after the tidal reversal on 23 May, changing to the higher tide on 25 May (Fig. 7). On this day both tides occurred in daylight. Once the animals switched to the afternoon tide they again continued to prefer this tide until after the tidal reversal on 6 June, switching their preference after both high tides occurred during the day. This pattern was repeated following the final tidal reversal of the season on 21 June. The animals thus showed a clear preference for the higher tide: more were seen in association with the higher tide in 44 of 51 observation intervals (lunar days). The seven exceptions occurred after the three reversals in tide height. One interpretation is that the first light of dawn signaled the time to switch to the opposite tide.

A model for the regulation of mating activity in Limulus

From the evidence presented here, and our knowledge about the visual system of *Limulus polyphemus,* we propose the following model for the regulation of mating activity in this species. The initiation of mating activity at the beginning of the season

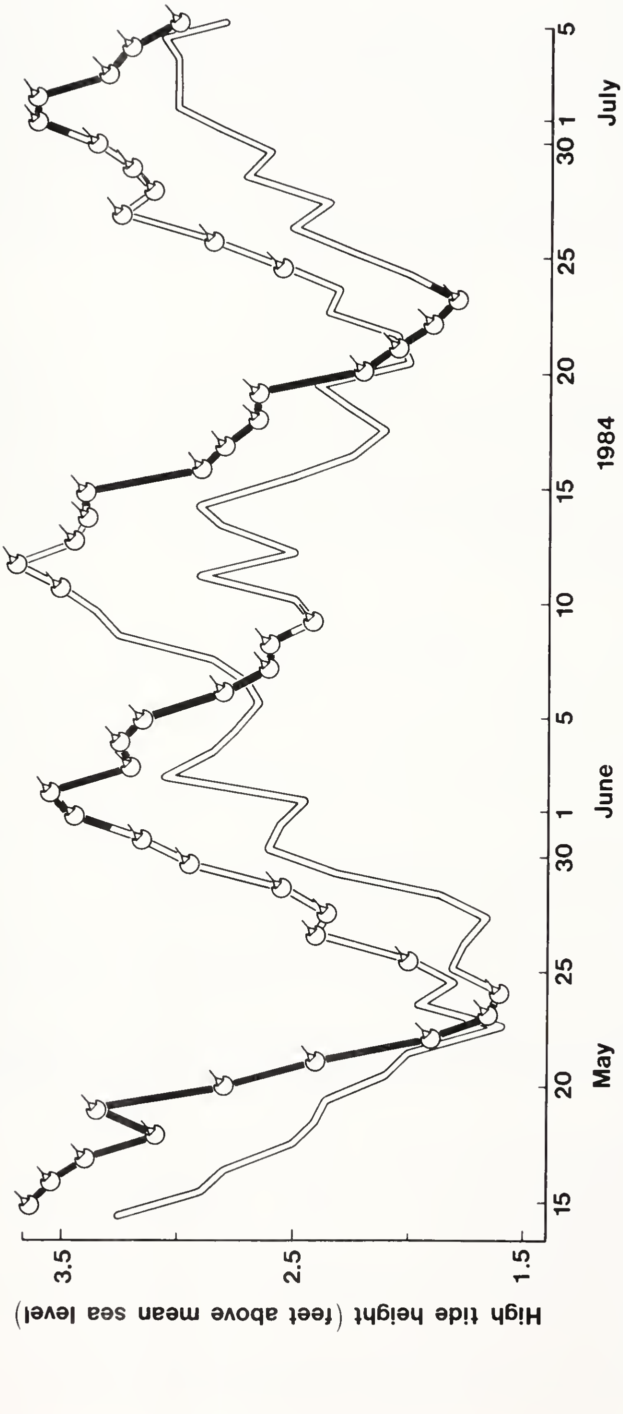

FIGURE 10. Summary of *Limulus* mating behavior during the 1984 season at Mashnee Dike. The height of each of the two daily high tides is tracked through the season by paths. The ordinate gives the tide height and the shade of each path indicates whether the high tide occurred in darkness or in daylight (including dawn and dusk). *Limulus*-shaped symbols mark the tide that was populated by the majority of horseshoe crabs.

is determined by changes in daylength and/or water temperature that occur in the spring. Detection of daylength may involve an endogenous circadian oscillator that continually resets its duty cycle with the changing photoperiod. The first high tide that the animals follow into the intertidal zone is determined by sensing its relative height. Once this is determined, the majority of animals move in on alternate high tides, showing a clear preference for the highest tide. Alternatively, they may continue to try to discriminate tide height, becoming progressively worse as the tides become more equal in height. This behavior persists until several days after the reversal in tidal inequality, when small but nearly equal numbers of animals appear during each high tide. Ultimately dawn occurs coincident with the populated tide and signals the animals that the opposite high tide has become higher. They then switch and begin migrating to the intertidal zone with the higher tide.

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MAGNESIUM DEPENDENCE OF ENDOTOXIN-INDUCED DEGRANULATION OF *LIMULUS* AMEBOCYTES

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ABSTRACT

Amebocytes are blood cells that function in the defense system of the horseshoe crab *Limulus polyphemus.* When they are withdrawn from the animal, they flatten and degranulate within hours. We have found, however, that when a small amount of blood is drawn into a syringe containing a large amount of 3% NaCl, free of divalant cations, and further diluted (to a final concentration of approximately 1:500) in 3% NaCl into tissue-culture-treated plates, amebocytes do not flatten or degranulate, even in the presence of endotoxin. This dilution technique was used to determine direct effects of Ca^{++} and Mg^{++} in the flattening and degranulation of amebocytes.

Ca^{++} or Mg^{++} added at normal hemolymph concentration to 3% NaCl caused rapid flattening. Degranulation did not occur for several days. In the presence of nanogram quantities of endotoxin, cells in Ca^{++} fortified saline *remained granulated,* while those in Mg^{++} fortified saline completely *degranulated* within several hours. Experiments with EGTA and EDTA confirmed that Ca^{++} is not essential for degranulation of amebocytes in the presence of serum. Although Ca^{++} and Mg^{++} are both influential in the *in vitro* flattening of *Limulus* amebocytes, Mg^{++} is essential for the process of degranulation.

INTRODUCTION

Granular amebocytes are the major blood cells of *Limulus polyphemus* and play an important role in the defense system. In the presence of endotoxin, factors of the extracellular gelation system are released from amebocytes. The resultant extracellular gel presumably immobilizes bacteria (Bang, 1956; Levin and Bang, 1964, 1968; Levin, 1976; Armstrong and Rickles, 1982). In the absence of endotoxin, amebocytes in culture are motile (Armstrong, 1977, 1979, 1980) and are able to phagocytose (Armstrong and Levin, 1978). In the absence of anti-aggregating chemicals such as *N*-ethylmaleimide (Solem, 1970), EDTA (Kenney *et al.,* 1972; Armstrong, 1980) propranolol (Murer *et al.,* 1975), and methylxanthine derivatives (theophilline, caffeine, theobromine) (Kobayachi and Yamamoto, 1974), amebocytes *in vitro* flatten and degranulate (Armstrong and Levin, 1978; Armstrong 1977, 1979, 1980). In order to understand better the activation of amebocytes, a modified dilution technique that prevents flattening and degranulation of amebocytes *in vitro* without the necessity for the addition of anti-aggregating chemicals was developed. Using this technique, we observed the independent effects of Mg^{++} and Ca^{++} on flattening and endotoxin-induced degranulation of amebocytes. We found that, although both Mg^{++} and Ca^{++}

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caused flattening of amebocytes, only Mg^{++} was essential for endotoxin-induced degranulation.

MATERIALS AND METHODS

Animals

Limulus polyphemus were obtained throughout the year by trawl from high salinity waters (ranging near 1000 kOs) from 10°C to 29°C within three miles of the Beaufort Inlet on the eastern shore of North Carolina. The animals were maintained in outdoor, tidal-exchanged, natural-bottomed, flow-through pens, (25 feet by 25 feet, containing a maximum of 200 animals at any given time), where they were able to feed at will. No exogenous food was added. One to three days before experimentation, the animals were moved to indoor tanks of flowing seawater and maintained unfed until use.

Reagents

Water used in experiments was sterile for injection (Abbott Laboratories, North Chicago, Illinois). Endotoxin was *Escherichia coli* UKT-B lipopolysaccharide (WAKO Pure Chemical Industries, Ltd., Osaka, Japan). Potency of the endotoxin was confirmed with U.S.P. Reference Standard Endotoxin EC-5 by the Limulus Amebocyte Lysate test. The potency of this preparation was 25 EU/ng. One thousand EU of this preparation contained less than 0.005 μg of Mg^{++} and less than 0.02 μg of Ca^{++} as measured by atomic absorption. Sterile Trypan Blue (Gibco, Grand Island, Maine) was made isotonic by addition of sodium chloride. Other chemicals were reagent grade. Inorganic chemicals were rendered pyrogen-free by baking at 180°C–210°C for 4 h.

Equipment

Sterile Linbro tissue-culture-treated, multi-well plates, (Cat. No. 76-033-05), sterile Linbro non-tissue-culture-treated, multi-well plates (Cat. No. 76-258-05, Flow Laboratories, McLean, Virginia), plastic, 60 × 15 mm non-tissue-culture-treated Petri dishes (Falcon #1007, Oxnard, California), 16 gauge, 25.4 mm needles (#5197), and 60 cc Plastipak syringes (#5663) (Becton-Dickenson, Rutherford, New Jersey) were used. Glassware, spatulas, and forceps were rendered pyrogen-free by baking at temperatures of 180°C–210°C for 4 h. Differential interference contrast, phase, and brightfield photomicrography was employed; results were recorded on Kodak Panatomic-X 32 film (Eastman Kodak, Rochester, New York).

Bleeding animals

To obtain amebocytes, we cleansed the flexure of the animals' prosoma and opisthosoma with 70% ethanol and withdrew 1 to 2 ml of hemolymph by cardiac puncture with sterile 16 g needles attached to sterile 60 cc syringes containing 49 ml of pyrogen-free 3% NaCl.

Preparation of serum

Bulk hemolymph for serum preparation, obtained by cardiac puncture with a 13 gauge needle, was allowed to clot overnight at 4°C and was centrifuged at 2000 rpm for 1 h to remove clotted amebocytes and cellular debris. Serum was stored until use at 4°C.

Preparation of amebocytes

Amebocytes were prepared for tissue culture experimentation as follows. Immediately after bleeding, approximately 50 to 100 μl hemolymph, diluted 1:50 with 3% NaCl, were added dropwise to tissue-culture-treated, multi-well plates containing an additional ml of 3% NaCl. Final cell concentration, determined by direct cell count with a hemocytometer, was approximately 4 to 5 × 10^4 per ml. Cells settled undisturbed for at least 1 h before experimentation.

Amebocytes were prepared for photomicrography by placing pyrogen-free coverslips in plastic Petri dishes. Cells settled onto the coverslips, which then could be removed for more effective photomicrography.

Surface effects and viability study

To determine the effects of various surfaces on amebocytes, cultures in 3% saline were tested on tissue-culture-treated, multi-well plates, non-tissue-culture-treated plastic Petri dishes, glass Petri dishes, and siliconized-glass Petri dishes. Trypan blue was used as a viability stain (Merchant *et al.*, 1964). Cells were placed in 3% NaCl into all the wells of a tissue-culture-treated, multi-well plate. Cells were kept unfed at ambient temperature and conditions. One well of cells per day was tested for viability.

Ca^{++} and Mg^{++} studies

Amebocytes were placed into multi-well, tissue-culture-treated plates as above. After the cells had settled, supernatant was removed with a sterile pipet and was replaced with isotonic saline containing calcium chloride at 0.24 m*M* to 15.0 m*M* or magnesium sulfate at 3.30 m*M* to 75 m*M*. Endotoxin was added at final concentrations of 10–2500 EU/ml to cells at all of the concentrations of Mg^{++} and Ca^{++} studied. Within 24 h of addition of Mg^{++} and Ca^{++}, coverslips with experimental cells were removed from dishes and placed uncovered on slides. Results were recorded by photomicrography with differential interference contrast optics.

EDTA and EGTA studies

Amebocytes were placed into tissue-culture-treated, multi-well plates. EDTA at 20 m*M* and 40 m*M* or EGTA at 5 m*M* and 10 m*M* was added to the serum. NaCl was removed from prepared cells and replaced with serum alone or serum containing either EDTA or EGTA at the above concentrations. Results were recorded within 24 h by photomicrography.

RESULTS

Development of dilution technique

Substrates were tested for their effects on amebocyte morphology. Amebocytes attached to tissue-culture-treated, multi-well plates but did not flatten or degranulate

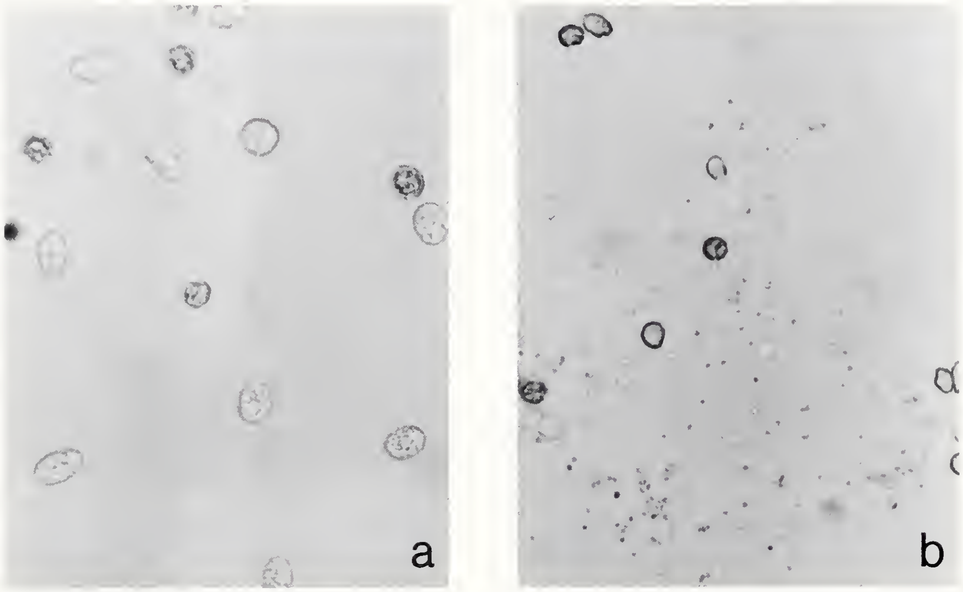

FIGURE 1. Effects of surfaces on *Limulus* amebocytes cultured for 1 h in 3% NaCl. (a) Sterile Linbro tissue-culture-treated, multi-well plates; cells are ovoid and granulated. (b) Sterile Linbro non-tissue-culture-treated, multi-well plates; intact granules released from lysed cells. Brightfield photomicrographs, ×200.

until death (Fig. 1a). Trypan blue exclusion tests indicated that these cells remained alive for approximately one week. In non-tissue-culture-treated plastic dishes, many cells disintegrated upon contact with the surface and others attached but contracted and became generally smaller and more rounded. Granules from cells that disintegrated remained intact (Fig. 1b). On siliconized glass surfaces, amebocytes attached and contracted with some disintegration similar to non-tissue-culture-treated plastic plates. Cells on non-siliconized glass Petri dishes attached and remained ovoid with some slight contraction and rounding similar to those in tissue-culture-treated, multi-well plates. Tissue-culture-treated, multi-well plates were, therefore, used experimentally because they affected cellular morphology least.

Seasonal variation was observed in the reactivity of amebocytes and their responses to surfaces. During summer months, amebocytes appeared to respond to stimuli (serum or Mg^{++}) more quickly and looked "healthier" (more regularly shaped) than did amebocytes obtained during winter months. This phenomenon did not alter the results reported.

Experiments

Amebocytes cultured in 3% NaCl remained ovoid and granulated. Amebocytes cultured in 3% NaCl and exposed to 50 m*M* Mg^{++} (Fig. 2a) flattened completely and remained granulated for 24 h. The process of flattening is begun in the presence of 6.25 m*M* Mg^{++} and is complete at concentrations of 25 m*M* Mg^{++} and above. A similar reaction was observed with 10 m*M* Ca^{++}. The process of flattening is begun in the presence of 0.5 m*M* Ca^{++} and is complete at concentrations of 10 m*M* Ca^{++}

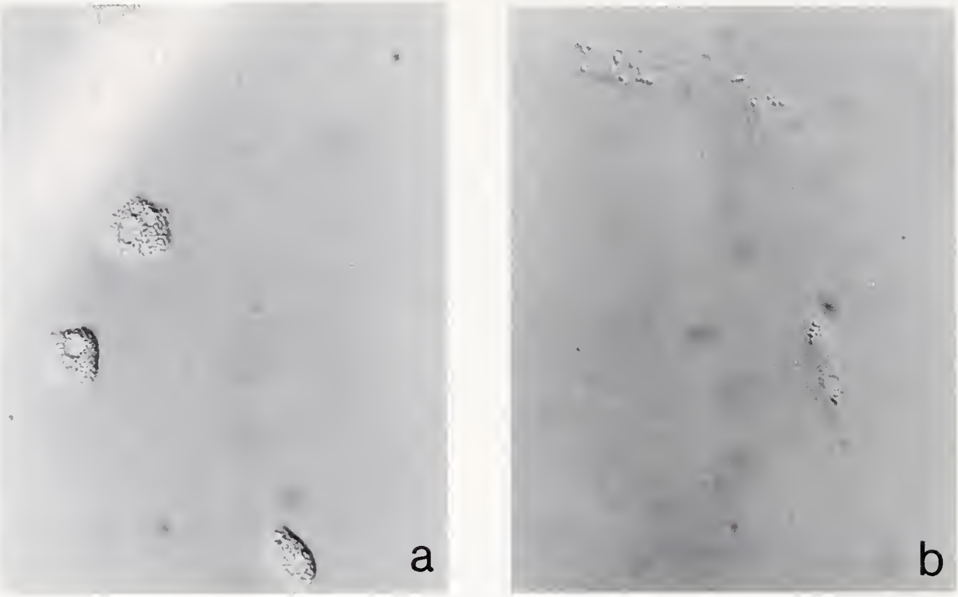

FIGURE 2. Effects of endotoxin on *Limulus* amebocytes cultured for 6 h in 3% NaCl containing 50 m*M* Mg^{++} on glass coverslips. (a) 3% NaCl containing 50 m*M* Mg^{++} without endotoxin; cells are flat and granulated. (b) 3% NaCl containing 50 m*M* Mg^{++} in the presence of 125 EU/ml endotoxin; cells are flat and degranulated. Differential interference contrast photomicrographs, ×400.

and above. Various transitional stages of flattening are seen between 6.25 and 25 m*M* Mg^{++} and between 0.5 m*M* and 10 m*M* Ca^{++}.

Endotoxin at 2500 EU/ml added to amebocytes cultured in 3% NaCl did not cause degranulation. Amebocytes remained ovoid and granulated. When 125 EU/ml endotoxin were added to amebocytes in the presence of 50 mM Mg^{++}, degranulation was complete within 24 h (Fig. 2b). Mg^{++}-facilitated degranulation as a function of endotoxin concentration is shown in Table I. The effects of varying concentrations of Mg^{++} in the presence of constant amounts of endotoxin are shown in Table II. The amount of endotoxin necessary to produce degranulation was dependent on Mg^{++} concentration. A minimum of 25 m*M* Mg^{++} was required for the degranulation of amebocytes in the presence of 2500 EU/ml of endotoxin. When endotoxin in concentrations ranging from 10 to 2500 EU/ml was added to amebocytes in the presence of 0.1 to 15 m*M* Ca^{++}, the amebocytes remained granulated.

TABLE I

Mg^{++} supported degranulation of amebocytes in the presence of endotoxin

	Endotoxin (EU/ml)							
Mg^{++} (m*M*)	0.0	15.6	31.2	62.5	125	250	1000	2500
0	+	+	+	+	+	+	+	+
50	+	+	+	±	−	−	−	−

+ = granulated, ± = partially granulated, − = degranulated.

TABLE II

Endotoxin-induced degranulation of amebocytes in the presence of Mg^{++}

Endotoxin (EU/ml)	Mg^{++} (mM) 0.0	0.5	3.1	6.2	12.5	25.0	50.0
0	+	+	+	+	+	+	+
1000	+	+	+	+	±	±	−
2500	+	+	+	±	±	−	−

+ = granulated, ± = partially granulated, − = degranulated.

Amebocytes cultured in 10 to 100% *Limulus* serum flattened and also degranulated within 24 h. EDTA at 20 mM and 40 mM in hemolymph serum prevented amebocytes from flattening and degranulating. However, amebocytes exposed to 5 mM and 10 mM EGTA-supplemented serum flattened and degranulated within 24 h.

DISCUSSION

Amebocytes, when placed on negatively charged surfaces, (glass and tissue-culture-treated, multi-well plates), in 3% NaCl, attached but retained the *in vivo* characteristics of ovoid shape and granule retention for one week (Fig. 1a). The phenomenon of amebocytes disentegrating with intact granule release when placed on hydrophobic surfaces (siliconized glass and non-tissue-culture-treated, plastic dishes) (Fig. 1b) may be a result of interaction between these surfaces and cell membranes. The technique of obtaining amebocytes with few morphological changes in the absence of anti-aggregating chemicals will be useful for future studies on amebocyte activation.

Partial flattening and spontaneous degranulation of amebocytes on artificial surfaces have been reported (Armstrong, 1980). Divalent cations and unknown hemolymph components have been considered to be causal factors in these cellular modifications (Armstrong, 1980). In this study, flattening of amebocytes cultured in 3% NaCl resulted from the addition of Mg^{++}, Ca^{++}, or hemolymph serum. Spontaneous degranulation followed the addition of hemolymph serum. To prevent or delay these reactions without anti-aggregating chemicals, immediate dilution by a factor of at least 500 was necessary. Amebocytes obtained by this method, although not in an *in vivo* state, had not begun the processes of flattening and degranulation.

Amebocytes cultured in 3% NaCl began to flatten in the presence of greater than 12.5 mM Mg^{++} or 1.0 mM Ca^{++}. In the absence of endotoxin these amebocytes retained their granules for more than 24 h.

Endotoxin causes the gelation of *Limulus* hemolymph (Levin and Bang, 1964). Gelation follows the exocytosis of amebocyte granules, which contain all of the factors of the gelation system (Murer *et al.*, 1975; Ornberg and Reese, 1981; Armstrong and Rickles, 1982). No triggering mechanism for exocytosis has been reported. As a result of the dilution technique, endotoxin could be added in amounts as great as 2500 EU/ml with *no resultant degranulation* of amebocytes cultured in 3% NaCl. This failure to degranulate indicates that degranulation requires the presence of a factor other than endotoxin. Our studies indicate that Mg^{++} is that factor. In the presence of 50 mM Mg^{++}, degranulation occurred rapidly among amebocytes when more than 125 EU/ml endotoxin were added (Table I). Higher concentrations of endotoxin required less Mg^{++} for degranulation to occur (Table II). Degranulation of amebocytes did not occur in the presence of calcium even at a concentration of 2500 EU/ml endotoxin. Therefore, Mg^{++}, but not Ca^{++}, is essential for endotoxin-induced degranulation. Although magnesium-dependent exocytosis may not be unique in nature, we have found no similar reports. This presents a challenge to examine more closely the role of Mg^{++} as a trigger for biological events.

The observation that EDTA inhibited spontaneous degranultion of amebocytes in serum and EGTA did not inhibit degranulation supports the assertion that Ca^{++} *is not* necessary for the degranulation of amebocytes. EDTA chelates both Mg^{++} and Ca^{++} while EGTA chelates Ca^{++} but does not chelate Mg^{++}. Spontaneous degranulation occurs within hours in amebocytes cultured in hemolymph (Armstrong, 1980, 1982). When cells were cultured by the dilution technique, complete degranulation was delayed. Eventual degranulation may be due to residual amounts of Mg^{++} and other serum factors added as part of the original innoculum.

Aggregation of amebocytes and flattening and degranulation of amebocytes are separable processes. There is a serum factor that causes aggregation even in the presence of EDTA (Kenney *et al.,* 1972). Our observations indicate that EDTA inhibits both flattening and degranulation even in the presence of hemolymph that contains this aggregating factor.

Limulus hemolymph contains 46 mM Mg^{++} and 10 mM Ca^{++} (Prosser, 1973), sufficient amounts to cause flattening of amebocytes in culture. However, amebocytes not only flatten but also degranulate in the presence of serum even in the absence of endotoxin; and since Mg^{++} and Ca^{++} do not cause degranulation, the presence of a "degranulation-promoting" factor in hemolymph serum is indicated. It appears that spontaneous degranulation, described by Armstrong (1982), is caused by this "degranulation-promoting" factor present in hemolymph serum. This factor may be released from tissues or amebocytes when *Limulus* has been injured or irritated.

Our observations that endotoxin in the presence of Mg^{++} causes degranulation and that degranulation occurs in the presence of serum in the *absence* of endotoxin suggest two possible series of events. The *degranulation-promoting* factor may have binding sites analogous to those of the lipopolysaccharide molecule and, therefore, may activate amebocytes to degranulate through the same pathway or mechanism. Alternatively, there may be two separate mechanisms that initiate amebocyte degranulation. One pathway may involve the reaction of Mg^{++} with lipopolysacharride leading to degranulation since the presence of Mg^{++} appears to be necessary for degranulation to occur. A second pathway may involve separate reactions, involving the *degranulation-promoting* factor, that may lead to a link with the lipopolysaccharide/Mg^{++} pathway. Further research is needed to test these hypotheses. Another area of possible future research involves the question of whether Mg^{++} acts intracellularly or extracellularly.

ACKNOWLEDGMENTS

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ASSOCIATION OF CENTRIOLES WITH THE MARGINAL BAND IN SKATE ERYTHROCYTES

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ABSTRACT

Centrioles are associated with the marginal bands (MBs) in certain invertebrate erythrocytes, functioning as organizing centers for MB reassembly (Nemhauser *et al.*, 1983). However, a similar association has not been observed previously in erythrocytes of vertebrates. Detergent-lysed erythrocytes ("cytoskeletons") of the skate *Raja erinacea* contain centrioles visible as paired dense dots in phase contrast. In uranyl acetate-stained whole mounts and in thin sections (TEM) they exhibit typical right-angle orientation and 9-triplet ultrastructure. Although the centriole pairs in some cytoskeletons are distant from the MB, surveys of their distribution in preparations from different animals indicates that it is non-random, with the majority adjacent to the MB or less than 1 μm from it. Many of the centriole pairs appear to be attached to MB microtubules, or have microtubules extending from them toward the MB. In rare instances, pointed cytoskeletons are observed with the centrioles at the apex from which fibers radiate, suggesting a morphogenetic function. The observations support the possibility that centrioles function during MB biogenesis in differentiating vertebrate erythrocytes, with loss of functional location as the cells mature.

INTRODUCTION

Comparative studies of cytoskeletal structure in blood cells of vertebrates and invertebrates have shown that marginal bands (MBs) of microtubules are prominent components of erythrocytes and clotting cells throughout the animal kingdom (Meves, 1911; Fawcett and Witebsky, 1964; Behnke, 1970; Goniakowska-Witalinska and Witalinksi, 1976; Cohen and Nemhauser, 1985). In erythrocytes, MBs first appear during cellular morphogenesis and are believed to function in bringing about the transformation from spherical to flattened discoid or elliptical cell shape (Barrett and Scheinberg, 1972; Small and Davies, 1972; Barrett and Dawson, 1974; Yamamoto and Iuchi, 1975). Recent experiments on vertebrate and invertebrate erythrocytes indicate that MBs continue to function in mature cells, resisting shape changes and/or restoring cell shape after deformation by external forces (Joseph-Silverstein and Cohen, 1984, 1985).

Although the circumferential location of MB microtubules in the plane of cell flattening is of considerable interest with respect to spatial control of microtubule arrays, relatively little is known of the mechanisms involved in MB formation. In mature chicken erythrocytes there may be structural or molecular "tracks" along the inner cell surface which guide the growth of MB microtubules during experimentally

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Abbreviations: PIPES = piperazine-n-n'-bis (2-ethane sulfonic acid), EGTA = ethyleneglycol-bis-(b-aminoethyl ether) n n'-tetraacetic acid, TAME = p-tosyl arginine methyl ester HCl, MB = marginal band, SAC = cell surface-associated cytoskeleton, PMSF = phenylmethylsulfonyl fluoride.

induced MB reassembly (Granger and Lazarides, 1982; Miller and Solomon, 1984), and in chick bone marrow erythroblasts centrosomes may play a role at an early stage of MB formation (Murphy *et al.,* 1986). In certain invertebrate ("blood clam") erythrocytes, MB-associated centrioles serve as organizing centers during temperature or taxol-induced MB reassembly (Cohen and Nemhauser, 1980; Nemhauser *et al.,* 1983; Joseph-Silverstein and Cohen, 1985), and MB-associated centrioles have also been observed in sea cucumber erythrocytes (Fontaine and Lambert, 1973). However, a similar structural relationship has not been reported previously for mature (circulating) erythrocytes of any vertebrate.

The purpose of this paper is to show that, for one vertebrate at least, such an association does exist. In the skate, *Raja erinacea,* most of the erythrocytes contain readily identifiable centriole pairs, a majority of which are adjacent to the MB.

MATERIALS AND METHODS

Skates (*Raja erinacea*) were provided by the Department of Marine Resources of the Marine Biological Laboratory, Woods Hole, Massachusetts. The animals were maintained in running seawater at MBL or in cooled (10°C), aerated tanks of "Instant Ocean" artificial seawater (Aquarium Systems, Inc., Eastlake, Ohio) at Hunter College. In the latter case "trace elements" (Hawaiian Marine Imports, Inc., Houston, Texas) were added to the water periodically.

Small samples of blood were obtained by snipping the tail tip, and larger ones by heart puncture after anesthesthetizing the animals in 0.04% tricaine in seawater. "Cytoskeletons" for routine examination and scoring of centriole location under phase contrast, were prepared by dilution of blood or cell suspensions approximately 1:10 into Triton lysis medium consisting of 100 m*M* PIPES, 5 m*M* EGTA, 1 m*M* $MgCl_2$, pH 6.8 (=PEM) containing 10 m*M* TAME and 0.4% Triton X-100. This medium had been used previously for studies of cytoskeletal structure in blood cells of diverse species (Cohen, 1978; Cohen *et al.,* 1982; Cohen and Nemhauser, 1985). In one experiment, 0.1% glutaraldehyde was included in the lysis medium to achieve simultaneous lysis and fixation (Cohen and Nemhauser, 1980). Cell morphology was observed in phase contrast in both living cells and cells fixed in Elasmobranch Ringer's (Cavanaugh, 1975) containing 0.1% glutaraldehyde so as to avoid artifacts due to contact with glass slides and coverslips.

Cytoskeleton whole mounts for transmission electron microscopy were prepared on Formvar-coated grids. The Formvar surface was pre-treated with polylysine (1% solution of MW > 400,000, followed by water washes and air drying) to enhance retention of material (Mazia *et al.,* 1975). Cytoskeleton suspensions in Triton lysis medium were placed on grids for 5 min, followed by a wash in PEM and 10 min fixation in PEM containing 2% glutaraldehyde. Subsequently, grids were washed in PEM and in water, stained with 1% aqueous uranyl acetate, and air-dried.

Material was prepared for thin sectioning as follows: 0.3 ml packed washed cells were suspended in 6 ml Brij lysis medium, consisting of PEM containing 0.6% Brij 58, 10 m*M* TAME, and 0.1 m*M* PMSF (freshly added). Brij was used because it produced less twisting of cytoskeletons than Triton, and also permitted easy resuspension of cytoskeletons. The material was centrifuged for 1 minute at top speed in the International Clinical centrifuge (2250 × *g*), resuspended as before and centrifuged again. The cytoskeletons were resuspended and fixed 1 h at room temperature in 6 ml PEM containing 2.5% glutaraldehyde. They were sedimented, washed once in 6 ml PEM by resuspension and centrifugation, postfixed for 1 h in 1% OsO_4 in PEM, washed three times in PEM, dehydrated in ethanol, and embedded in Epon.

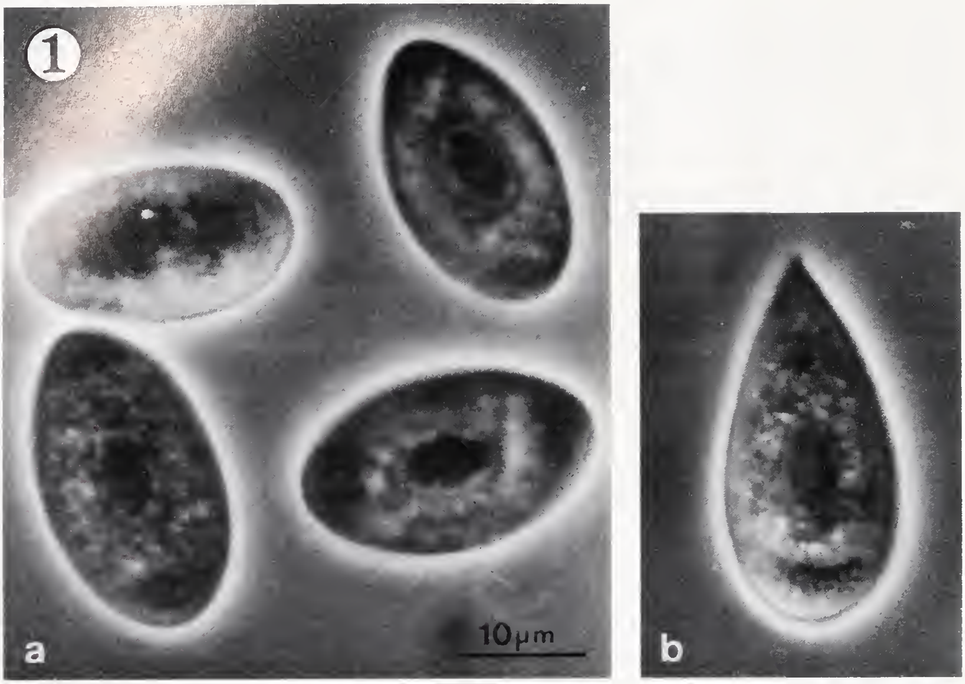

FIGURE 1. Erythrocytes of the skate, *Raja erinacea,* exhibiting the nucleated, flattened, elliptical morphology typical of all non-mammalian vertebrates (a). On rare occasion, cells with single or double-pointed shape are observed (b). Phase contrast.

Thin sections were cut with diamond knives on the Sorvall MT-2 ultramicrotome (DuPont Instruments, Newtown, Connecticut), and stained with saturated uranyl acetate in 50% ethanol followed by Reynold's lead citrate. Whole mounts and thin sections were examined in the Hitachi HS-8 (50 kV) or Zeiss EM10C (80 kV) transmission electron microscopes.

RESULTS

In both fresh blood samples and samples of washed, fixed cells, the erythrocytes of the skate, *Raja erinacea,* are found to be morphologically similar to those of most non-mammalian vertebrates (Fig. 1a). They are nucleated, flattened, and, with rare exception, elliptical, with the long axis in the 20–25 µm range. This is a relatively large size for fish in general, but typical of elasmobranchs as compared with teleosts (Andrew, 1966; Nemhauser *et al.,* 1979). Cells with single or double-pointed shape are rarely observed morphological variants (Fig. 1b).

Skate erythrocyte cytoskeletons, prepared by lysis of the cells with Triton X-100 under conditions previously observed to stabilize MBs, are shown in Figure 2. In phase contrast the centrioles, verified as such by electron microscopy (see below), appear as paired dense "dots," and usually they are readily visible in flat (untwisted) cytoskeletons because there is little competing cytoplasmic structure. In many cases the centrioles are very close to and possibly in contact with the MB. At higher magnifications, with flattening of cytoskeletons under the coverslip to enhance viewing,

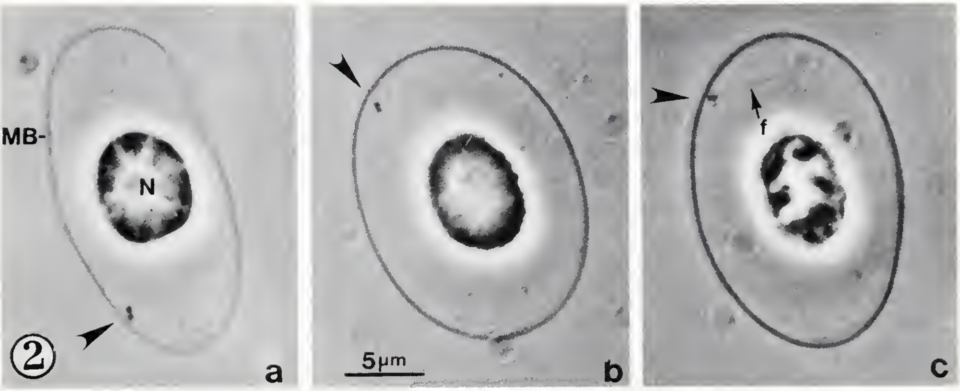

FIGURE 2. Skate erythrocyte cytoskeletons (Triton X-100 lysis), as observed in phase contrast under oil immersion. Centrioles appear as paired, phase-dense "dots" (arrowheads). (a) One of the centrioles very close to or in contact with MB; N = nucleus. (b) Centriole pair adjacent to, but not in direct contact with major part of MB; (c) centriole pair appearing to touch MB, with a "fiber" (f) extending away from it.

some of the centriole pairs are found to be attached to fibers (microtubule bundles) which are part of the MB, or which extend from the centrioles toward a distant point on the MB (Fig. 3).

Confirmation of the attachment of centrioles to the MB, including some which appeared separated from the main body of the MB, was obtained by examination of cytoskeleton whole mounts in TEM. In Figure 4 the centrioles are observed to be attached to only one or possibly a few microtubules of the spread MB. Figure 5 shows centrioles which, in phase contrast, would appear to be close to, but not touching the MB, but which are actually connected to it by radiating microtubules. Examination of the centrioles in underexposed prints revealed their cylindrical shape, microtubular substructure, and sometimes (as in Fig. 5) their approximately orthogonal orienta-

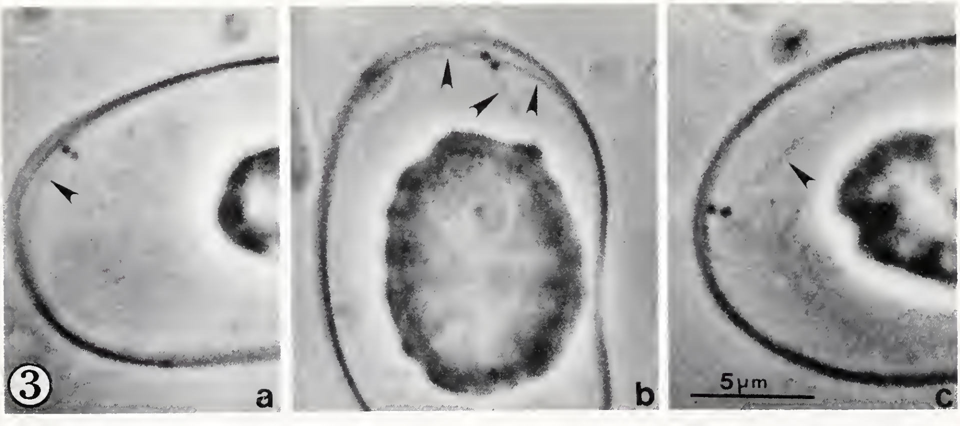

FIGURE 3. Cytoskeletons flattened under the coverslip to provide improved higher magnification views of centriole-associated fibers in phase contrast. Such fibers appear to be part of the MB (a, b; arrowheads), or to extend from at least one of the centrioles toward a distant point on the MB (c; arrowhead). Flattening also generally produced an artifactual increase in area of nucleus, as in b.

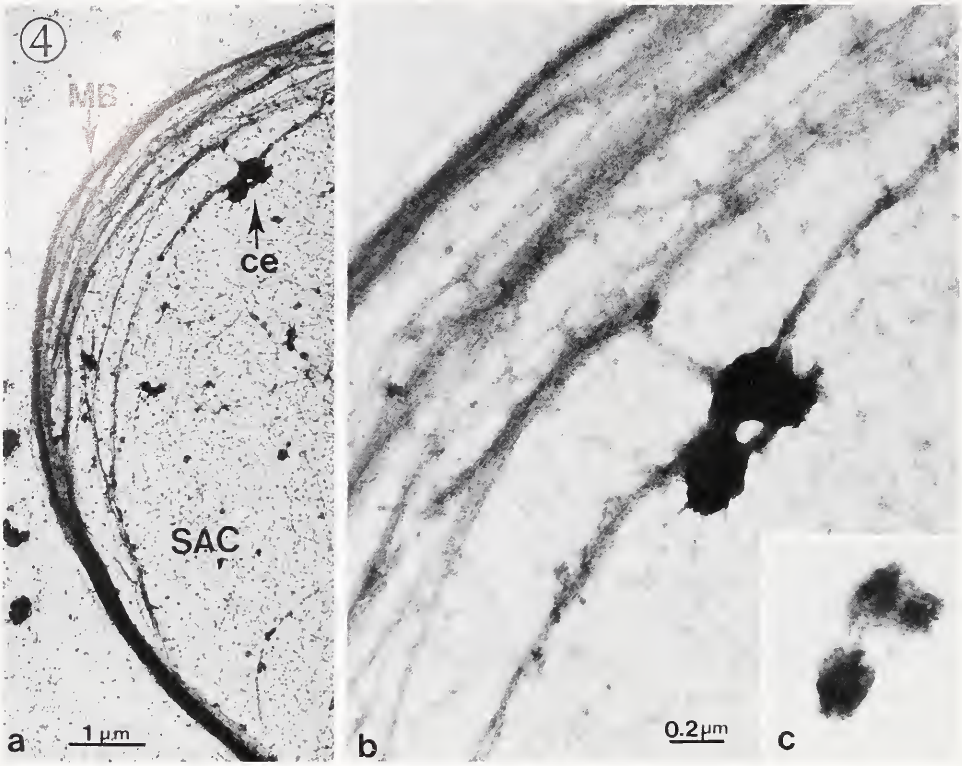

FIGURE 4. Skate erythrocyte cytoskeleton whole mount, uranyl acetate staining, TEM. (a) Survey view; the MB in this region has spread against the substratum; centrioles = ce. The "membrane skeleton," or "cell surface-associated cytoskeleton" (SAC), has collapsed onto the substratum and is visible as a background network. (b) Higher magnification view of centrioles in (a), revealing that the centriole pair is attached to one, or at most a few, MB microtubules. (c) Underexposed print of centrioles in b, in which centriolar substructure can be detected.

tion. Thin sections confirmed that they were typical centrioles, about 0.2×0.35 μm, with "9 + 0" substructure (Fig. 6).

While most cytoskeletons contained centrioles adjacent to or near the MB, in some the centrioles were located between MB and nucleus, while in others they were adjacent to the nucleus. Figure 7 illustrates a case in which the centriole pair was closer to nucleus than to MB. In such cases, radiating microtubules usually were not evident. In all of the whole mounts examined by TEM, the cell "membrane skeleton" or "surface-associated cytoskeleton" (SAC) was visible as a background network throughout the region between nucleus and MB (Figs. 4, 5, 7).

Two cytoskeletons out of several thousand examined in phase contrast were pointed and incomplete at one end, with a curved MB at the other end. In these, the centrioles were located within the pointed tip, in a region from which fibers radiated toward the closed end of the MB (Fig. 8). Though rare, these cytoskeletons gave the impression that the centrioles were active at one "pole" as organizing centers for a forming MB.

Upon casual inspection, the spatial distribution of centrioles appeared to be non-random, with most centriole pairs close to or touching the MB. This was confirmed

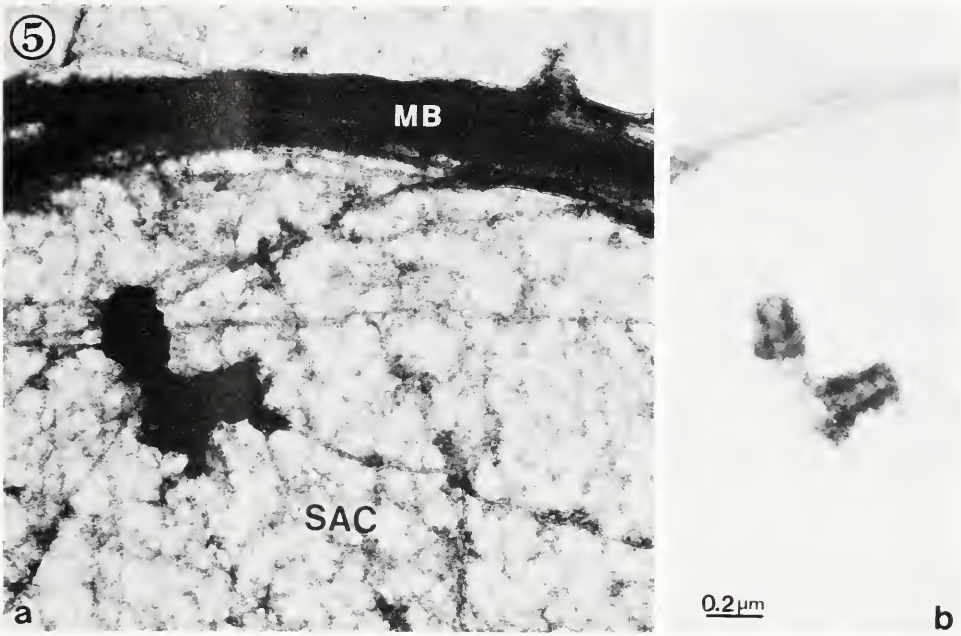

FIGURE 5. Skate erythrocyte cytoskeleton whole mount, uranyl acetate staining, TEM. (a) Survey view; the MB in this region is compact (not spread as in Fig. 4), with radiating microtubules connecting the centriole pair to the MB. The cell surface-associated cytoskeletal network (SAC) also is visible. (b) Underexposed print of the centrioles, showing cylindrical structure and nearly right-angle orientation.

in a semi-quantitative way by careful oil immersion observation of large numbers of cytoskeletons from six animals (Table I). A tabulation procedure was devised to count MB-associated *versus* cytoplasmic centriole pairs, so as to provide a general picture of their distribution (Fig. 9). For each of the six skates, 50% or more of the centriole

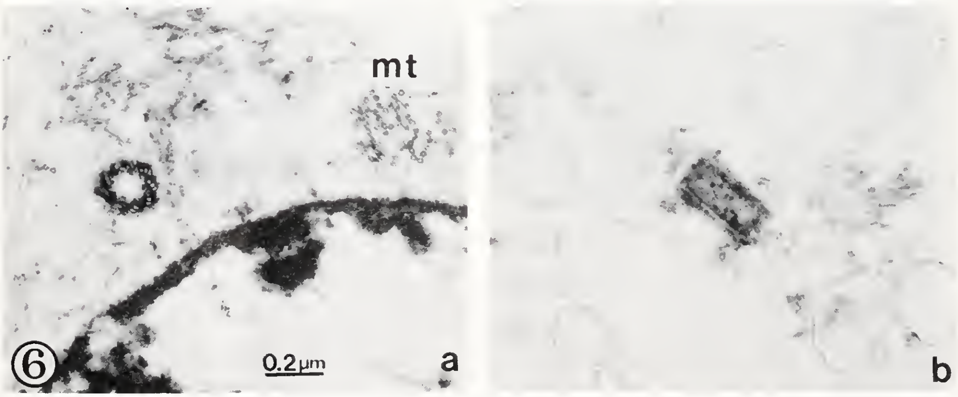

FIGURE 6. Skate erythrocyte centrioles as seen in thin sections of cytoskeletons, TEM. Typical "9 + 0" cross-sectional pattern (a) and cylindrical longitudinal structure (b) is observed. MB microtubules are also evident (mt).

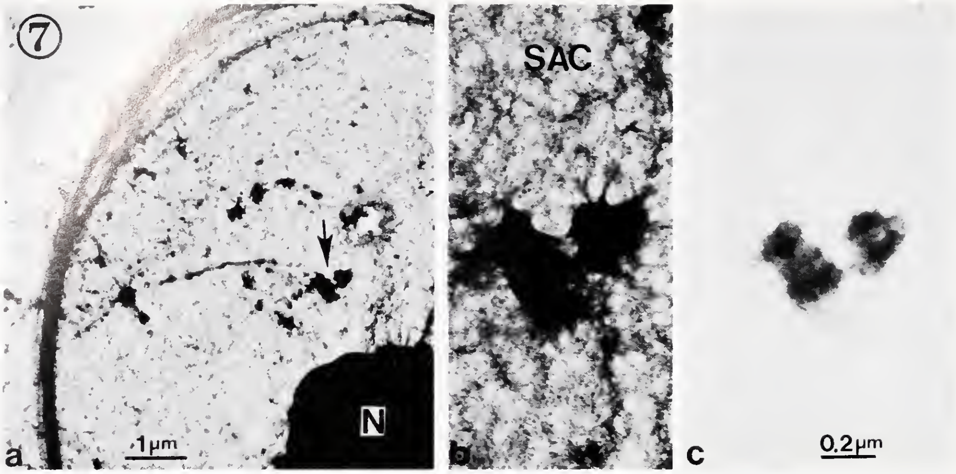

FIGURE 7. Skate erythrocyte cytoskeleton whole mount, uranyl acetate staining, TEM. Example in which the centriole pair is located closer to nucleus than to MB. (a) Survey view; centrioles at arrow; N = nucleus. (b) Higher magnification view of centrioles; few, if any, radiating microtubules are present. The centrioles are enmeshed between the two surface-associated cytoskeleton layers, which form a surrounding network (SAC). (c) Underexposed print of the centriole pair.

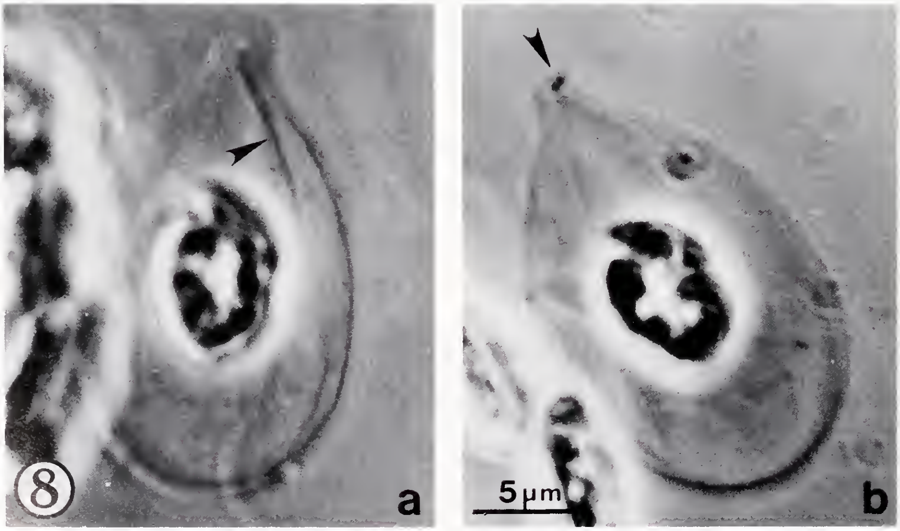

FIGURE 8. A rarely observed pointed skate erythrocyte cytoskeleton, as viewed in phase contrast under oil immersion. (a) One of several fibers (arrowhead) emanating from pointed (upper) region of cytoskeleton. These fibers radiate toward the distant closed end of the MB. (b) Different optical section of same cytoskeleton, showing the pair of centrioles present at the apex of this pointed region (arrowhead). (Note: material at left in photos is part of adjacent clump of cytoskeletons, and different orientation of cytoskeleton in b is due to its movement under coverslip between photographs.)

TABLE I

Distribution of centrioles in erythrocyte cytoskeletons prepared from different animals (phase contrast observations, oil immersion)

Skate no.[a]	No. cytoskeletons counted[b]	No. with centrioles adjacent to MB[c]	% Adjacent
1	104	70	67
2	107	65	60
3	100	80	80
4	100	52	52
5	103	52	50
6	100	55	55

[a] Blood samples were taken from skates during summer months at the MBL, except for #5, which was maintained at Hunter College at 10°C without feeding for more than two months before use.

[b] Triton lysis medium was used to prepare cytoskeletons for skates #1–5. For skate #6, Triton lysis medium containing glutaraldehyde was employed to achieve simultaneous lysis and fixation (see Materials and Methods).

[c] Criterion: at least one centriole of the pair within or touching outer zone of cytoskeleton (see Fig. 9).

pairs were within or touching an "outer zone" adjacent to the MB, representing less than 25% of the open area (excluding nucleus) of a cytoskeleton. Since the surface of these cells (as viewed on edge) tends to taper as it approaches the narrow perimeter containing the MB, the *volume* represented by the "outer zone" (Fig. 9) is actually less than 25% of that available. It was notable that in one of the skates, 80% of the centriole pairs were partially or wholly within this area (#3, Table I).

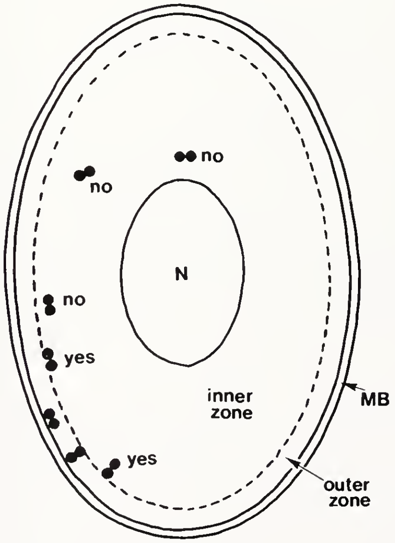

FIGURE 9. Diagram illustrating the counting procedure used to determine centriole distribution as reported in Table I. In addition to centrioles in contact with the MB, those within or touching the "outer zone" of a cytoskeleton were scored as adjacent to the MB (examples labelled "yes"); those in the interior ("inner zone") were scored as not MB-associated (examples labelled "no"). The "outer zone" was approximately one centriole pair (0.75 μm) in width, representing <25% of open area within MB (excluding nuclear area).

Discussion

Centrioles have been observed previously in the mature erythrocytes of several non-mammalian vertebrates, including amphibia (*Xenopus laevis, Rana catesbiana, Necturus maculosus*) and a reptile (*Stenotherus odoratus*) (Gambino *et al.,* 1982; Euteneuer *et al.,* 1985), and data on microtubule polarity in MBs of several of these species are consistent with a model involving initiation of microtubule growth at an organizing center (Euteneuer *et al.,* 1985). However, the centrioles are generally adjacent to the nucleus rather than associated with MBs in these vertebrates, consistent with the idea that MB biogenesis in vertebrate erythrocytes may involve other kinds of organizing centers and/or spatial determinants, such as peripheral morphological and/or molecular "tracks" (Granger and Lazarides, 1982; Miller and Solomon, 1984; Swan and Solomon, 1984).

The present observations on skate erythrocytes suggest a relationship between centrioles and MBs, raising the issue as to why significant numbers of MB-associated centrioles have not been observed in other vertebrates. One possibility is that the skate, as a relatively primitive, cartilagenous vertebrate, retains some primitive traits at the cellular level which have been lost in higher vertebrates. This is unlikely, because in the dogfish (their close elasmobranch relative) we have observed MB-associated centrioles relatively infrequently (Nemhauser *et al.,* 1979). Another possibility is that such an association is seasonal, disappearing except during periods of maximal erythropoiesis for a given species. The current study does not explore this question. Blood samples from skates #1–4 were obtained during the summer at a time when heightened growth and erythropoiesis might be expected; skate #5 (Table I) was maintained for >2 months at 10°C without feeding, yet its erythrocytes retained roughly 50% of centrioles near the MB. However, the significance of this observation cannot be determined at present, since it is unlikely that the treatment of skate #5 was physiologically equivalent to normal seasonal changes experienced in nature.

In considering the data on centriole distribution (Table I), it should be noted that centrioles cannot be studied readily by light microscopy in intact living or fixed erythrocytes, principally because they are obscured by hemoglobin. In addition, immunofluorescence techniques are not useful in fixed intact erythrocytes because the fixed hemoglobin forms a gelled matrix through which both antibody penetration and viewing are poor. Thus, examination of detergent-lysed cells (cytoskeletons) is the only convenient approach to studying large numbers of these cells. Such work requires the assumption that the distribution of centrioles in erythrocyte cytoskeletons reflects their location in living cells. The arguments that this is the case are as follows: (a) many of the skate erythrocyte centriole pairs are immediately adjacent to the MB. The assumption that they were not in that location prior to lysis requires their movement into the narrowest cell dimension, at the edge of the cell in the plane of flattening. Since the dimensions of a centriole pair (even without associated microtubules) approach that of the cell at this edge, such movement is highly unlikely. (b) In detergent-lysed erythrocytes of several other vertebrates which are known to contain centrioles, centrioles are not found distributed through the cytoplasmic region, but remain adjacent to the nucleus (Euteneuer *et al.,* 1985). (c) Immediately after cell lysis with Triton in both skate (this paper) and blood clam erythrocytes (Nemhauser *et al.,* 1983), without chemical fixation, centrioles are observed to be fixed in position. (d) When non-mammalian erythrocytes are lysed with Triton, the cell surface-associated cytoskeletal network (SAC; visible in Figs. 4, 5, 7) collapses inward from both sides as hemoglobin and other soluble components are released, trapping organelles within. This would be expected to prevent or at least inhibit marked movement of centriole pairs with associated microtubules. (e) In one experiment on the skate erythrocytes, glutaraldehyde was included in the Triton lysis medium, so that fixation would proceed simultaneously with cell lysis. Centriole distribution in these cytoskeletons was similar to that observed in the unfixed cytoskeletons (skate #6 *vs.* skates #1–5, Table I).

Although the observations on MB-associated centrioles reported here are probably the first for circulating erythrocytes of a vertebrate, the hemoglobin-containing erythrocytes of "blood clams" and certain sea cucumbers constitute well-documented prior cases among the invertebrates (Fontaine and Lambert, 1973; Nemhauser *et al.,* 1983). In the case of the blood clams, there is one centriole pair closely associated with each MB, and microtubule nucleating activity during experimentally induced MB reassembly initiates at, or in the vicinity of, these centrioles (Cohen and Nemhauser, 1980; Nemhauser *et al.,* 1983). The skate also has a large number of MB-associated centrioles, but the situation differs from that in blood clams. First, centrioles are not associated with every MB; as observed in cytoskeletons, they frequently occur distant to it in the open cytoplasmic region or adjacent to the nucleus. Second, there is considerable variation in centriole distribution in the erythrocytes of different skates (Table I). These observations are consistent with the following possibility: all vertebrate MBs may initially have centrioles/centrosomes associated as microtubule organizing centers during normal erythrocyte differentiation. As cells mature, centrioles might redistribute such that their location is a function of erythrocyte age. This could account for variation in numbers of MB-associated centrioles in different animals, reflecting erythrocyte age distribution in the circulating population and variations in hematopoietic activity prior to the time of blood sampling. A similar scenario has been suggested recently by Euteneuer *et al.* (1985) on the basis of data on MB microtubule polarity.

Although the centrioles are not randomly distributed in skate erythrocytes, centriole function with respect to the MB obviously remains to be demonstrated in this species. Unfortunately, under conditions tested thus far, the MBs are not cold-labile in living cells of *R. erinacea.* Thus it has not yet been possible to examine the role of centrioles in MB reassembly as done previously in the case of blood clam erythrocytes (Nemhauser *et al.,* 1983). However, the morphology of the pointed cytoskeletons (Fig. 8) is suggestive of centriole activity. Although only two such skate cytoskeletons have been observed to date, their appearance is similar to that observed in native pointed cytoskeletons of blood clam erythrocytes, and to stages in blood clam MB reassembly (Nemhauser *et al.,* 1983), in which centrioles and associated material constitute the nucleating "pole" for the MB. Is pointed morphology a normal stage in erythrocyte differentiation in the non-mammalian vertebrates? The literature does not answer this question, but pointed cells have been observed in the circulation of adult skates (Fig. 1), chickens (Lucas and Jamroz, 1961), and several other vertebrates.

In examining the possible role of centrioles in MB biogenesis, attention must be paid to possible differences between experimentally induced MB reassembly in mature erythrocytes and normal MB biogenesis during erythrocyte differentiation. Although centrosomes appear to be active in initial stages of MB biogenesis in chick bone marrow erythroblasts, they do *not* participate in experimentally induced MB reassembly in mature chicken erythrocytes (Miller and Solomon, 1984; Swan and Solomon, 1984; Murphy *et al.,* 1986). In addition, the MB in differentiating chicken erythroblasts contains approximately 50 microtubules, whereas that in mature chicken erythrocytes contains only about 12, and similar differences in microtubule numbers in differentiating *versus* differentiated erythrocytes have been observed for other species (Small and Davies, 1972; Yamamoto and Iuchi, 1975; Miller and Solomon, 1984). Thus, experimentally induced MB reassembly in a morphologically differentiated cell can provide only limited information about MB biogenesis. The role of centrioles and pericentriolar material in MB biogenesis must be properly assessed directly in the normally differentiating erythroblasts of various species, during the morphological transition from mitotic cell to flattened erythrocyte.

ACKNOWLEDGMENTS

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ATTACHMENT OF MATING REACTIVE *PARAMECIUM* TO POLYSTYRENE SURFACES: IV. COMPARISON OF THE ADHESIVENESS AMONG SIX SPECIES OF THE GENUS *PARAMECIUM*

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ABSTRACT

The relationship between mating reactivity and ability of cells to attach to polystyrene Petri dishes was investigated in six species of the ciliated protozoan *Paramecium.* Mating reactive-dependent attachment to polystyrene surfaces was seen in *P. caudatum, P. multimicronucleatum, P. tetraurelia,* and *P. trichium.* Attachment was rarely seen in either mating reactive or non-reactive cells of *P. bursaria* irrespective of extracellular ion concentration, temperature, and swimming velocity. Induction of attachment occurred in mating reactive cells of *P. bursaria* by treatment with trypsin. Strong affinity of cells for polystyrene surfaces was seen in *P. duboscqui* even when they were mating non-reactive or when applied to polystyrene dishes with reduced hydrophobicity. Induction of micronuclear activation by attachment—which was found in *P. caudatum*—was not observed in attached cells of the other species.

INTRODUCTION

Attachment of cells to polystyrene surfaces is a common phenomenon widely seen in various types of cells. In the ciliated protozoan *Paramecium caudatum,* attachment differs from that in other types of cells such as bacteria, slime molds, or cells in tissue culture of higher animals, since only mating reactive cells of *P. caudatum* can attach to the bottom of polystyrene dishes (Falcon 1007) and the attachment occurs only at the tips of ventral cilia where mating reactivity is restricted (Kitamura, 1982). An increase in affinity of cells for polystyrene occurs during the initial step of conjugation, which involves specific ciliary agglutination between cells of complementary mating types (Kitamura, 1984). This attachment, which seems to involve hydrophobic interactions, also induces the first step of nuclear activation (early migration of the gametic nucleus) and the subsequent loss of mating reactivity seen in normal conjugation. Thus, a series of hydrophobic interactions on the cell surface is believed to play an important role in the conjugation process of *P. caudatum.* Similar attachment was reported in another species, *P. multimicronucleatum,* though nuclear activation was not observed (Kitamura and Steers, 1983).

Conjugation of *Paramecium* is initiated by specific cell recognition between complementary substances on the cell surface, substances which are simple, non-conjugated proteins (Metz and Butterfield, 1951; Cohen and Siegel, 1963; Kitamura and Hiwatashi, 1978). It provides a good system for studying the problems of cell surface interactions since it is an extremely simple system to the extent that the interacting cells are structurally identical, the sexual interactions are strictly cell surface events,

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Abbreviation: EMM = early micronuclear migration.

and no diffusible substances are involved (Sonneborn, 1937; Metz, 1954; Hiwatashi, 1969, 1981; Miyake, 1981; Kitamura and Hiwatashi, 1984; Hiwatashi and Kitamura, 1985).

The polystyrene-attachment phenomenon may be useful to studying the cell surface interactions of *Paramecium*, since conditions for the attachment is very simple, *i.e.*, cells attach to polystyrene surfaces in simple salt solution and the attachment is not complicated by adsorption to the substratum of serum proteins which are usually added to tissue culture media. In addition, polystyrene surfaces are known to be used for the radioactive labelling of cell surface proteins (Chin and Lanks, 1980). Therefore, one means of studying specific interacting surface substances can employ use of artificial substrata of known structure and properties (Lanks and Chin, 1982).

This study employs such an approach using polystyrene surfaces to open the way to identify the cell surface substances involved in the conjugation process of *Paramecium*. I will describe first adhesiveness of cells to polystyrene dishes in six species of *Paramecium* to determine the relationship between mating reactivity and the ability to attach to polystyrene surfaces. Light microscopical studies also were performed to examine whether micronuclear activation is induced by attachment.

MATERIALS AND METHODS

Cells and culture methods

Ten stocks of syngen 3 of *P. caudatum*, 10 stocks of *P. multimicronucleatum*, syngen 2, 4 stocks of *P. tetraurelia*, 4 stocks of *P. trichium*, syngen 1, 20 stocks of *P. bursaria*, syngen 1, and 15 stocks of *P. duboscqui* were used. All the stocks of *P. multimicronucleatum* were homozygous for the acyclic allele in the expression of mating type (Barnett, 1966). Stocks of *P. multimicronucleatum* (stocks 103, 109, 203, and 204), *P. tetraurelia*, and *P. bursaria* were kindly provided by Dr. A. Barnett (University of Maryland), by Dr. S. Koizumi (Miyagi College of Education), and by Dr. H. Endoh (University of Tsukuba), respectively. Stocks of the CH-series in *P. multimicronucleatum*, *P. caudatum*, *P. trichium*, and *P. duboscqui* (original source, Dr. Shi Xin-Bai, Harbin Normal University) were obtained from the Hiwatashi collection at Tohoku University.

All the stocks were cultivated at 25°C in 1.25% (w/v) fresh lettuce juice medium diluted with Dryl's solution (Dryl, 1959) which was inoculated with *Klebsiella pneumoniae* one day before use (Hiwatashi, 1968). In cultures of all organisms except *P. bursaria*, several hundred cells were inoculated into an 18 × 180 mm test tube containing 2 ml of culture medium and expanded by adding fresh medium of 4 ml, 10 ml and 10 ml on successive days. Within one day after the last feeding the cultures reached the stationary phase of growth and most cells exhibited strong mating reactivity. The stocks of *P. bursaria* were cultured in test tubes at 25°C with a fixed illumination cycle of 12 h dark and 12 h light by adding 2 ml, 4 ml, 8 ml, and 8 ml of culture medium on successive days to reach stationary phase one day after the last feeding. After reaching the stationary phase, cultures of *P. bursaria* were left in a sunny room to maintain the diurnal rhythm of mating reactivity.

Measurement of attachment to polystyrene dishes

To measure cell attachment the previously reported method (Kitamura, 1982) was used. Cells were washed twice by centrifugation in a hand-operated centrifuge with a standard saline solution (1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1). The washed cells were equilibrated in glass test tubes with the same solution

TABLE I

Attachment of cells to the surface of polystyrene Petri dishes in P. caudatum, *syngen 3*

Stocks	Mating type	% Cells attached to the dishes[a]	
		Non-reactive cells[b]	Mating reactive cells
27aG3	V	3 (1–5)	79 (73–83)
Yt3-G1	V	2 (0.5–4)	80 (74–85)
StG1	V	1 (0.1–2)	67 (61–74)
Yt3-G9	VI	2 (0.5–4)	91 (87–94)
27aG3-6	VI	2 (0.5–4)	66 (60–72)
C103	VI	2 (0.5–4)	78 (72–84)
CHB	VI	0 (0–2)	21 (16–27)
CHB-s2	VI	0 (0–2)	23 (16–30)
CHB-s4	VI	0 (0–2)	9 (5–15)
CHB-s5	VI	0 (0–2)	18 (13–22)

[a] Cell suspensions of each stock in a medium that contained 1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1, were introduced into polystyrene dishes. Two minutes later, photographs were taken to measure attached cells. Numbers in parentheses show 90% confidence limits.

[b] Logarithmically growing cells in sexually mature period.

for 10 min at 21°C, after which 5 ml of the cell suspension containing about 1000 cells/ml was added to a 50 mm disposable polystyrene Petri dish (Falcon 1007). After 2 min the test dishes were photographed with a 1 s exposure and the percentage of cell attachment to the polystyrene surface was measured by counting between 100 and 200 cell tracks in each dish. The swimming velocity of cells was determined by measuring the length of about 20 tracks of photographs taken with a 2 s exposure. Cells measured for swimming velocity were placed in dishes for tissue-culture (Falcon 3002), in which attachment rarely occurred (Kitamura, 1982).

Observation of micronuclear changes

To test whether nuclear changes are induced by attachment to polystyrene, micronuclei of attaching cells were observed on preparations fixed by Carnoy's fixative (acetic acid/ethanol, 1:3) and stained with the Feulgen procedure.

RESULTS

P. caudatum

Only mating reactive cells of the *P. caudatum* stocks tested attached to Falcon 1007 polystyrene dishes. The attachment occurred only at the tips of ventral cilia, and attachment induced the first step of nuclear activation, *i.e.* early micronuclear migration (EMM). Unexpectedly, however, stock CHB showed a low ratio of attachment (about 20–30%) even when they were strongly mating reactive (Table I). The percentage of attached cells decreased to less than 10% within 5 min following introduction of cells to the dishes, suggesting a weak affinity of stock CHB cells for polystyrene surfaces. Three clones obtained from selfing progeny of stock CHB also showed a similar low percentage of attachment (9–23%) (Table I). However, it is uncertain whether stock CHB has a genetic defect in the mechanism of polystyrene attachment.

TABLE II

Attachment of cells to the surface of polystyrene dishes in P. multimicronucleatum, *syngen 2*

		% Cells attached to the dishes	
Stocks	Mating type	Non-reactive cells	Mating reactive cells
109	III	0 (0–4)	55 (44–66)
203	III	0 (0–4)	49 (42–57)
204	IV	1 (0.1–4)	52 (47–58)
103	IV	0 (0–4)	0 (0–4)
CH 104	III	0 (0–2)	48 (40–56)
CH 313	III	1 (0.1–2)	53 (44–63)
CH 100	III	0 (0–2)	45 (37–54)
CH 312	IV	0 (0–2)	52 (42–62)
CH 326	IV	0 (0–2)	36 (28–45)
CH 2	IV	0 (0–2)	52 (44–60)

Cells were washed with a solution containing 1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1, and equilibrated in the same solution for 10 min at 20°C.

P. multimicronucleatum

Mating reactive-dependent attachment to polystyrene was also observed in all of the tested stocks of *P. multimicronucleatum,* but only about 50% of cells attached even though more than 90% of the cells expressed mating reactivity. Moreover, cells of one unusual stock (stock 103) did not attach. In all experiments cells were cultivated in a 0.3% grass infusion (cerophyl) buffered with 0.1% Na_2HPO_4. Therefore, one may suspect that the ability of cells to attach to polystyrene was influenced by their growth in grass infusion. To test for this possibility, cells of the four stocks used in a previous study (Kitamura and Steers, 1983) plus six additional stocks were cultured in lettuce medium and attachment was tested. Table II shows that mating reactive-dependent attachment is highly reproducible in all stocks of *P. multimicronucleatum* except for stock 103 which showed no attachment even when mating reactive. Mating reactive cells attained a maximum attachment plateau within the first min following their introduction to the dishes (Fig. 1B). Notwithstanding that more than 90% of the cells had strong mating reactivity, approximately 50% attached, almost the same percentages as obtained with cerophyl cultured cells. These results demonstrate that there is no marked difference in attachment ability due to these culture conditions.

P. tetraurelia and P. trichium

Attachment to polystyrene surface was tested in four stocks of *P. tetraurelia* and four stocks of *P. trichium,* syngen 1. All of them showed mating-reactive dependent attachment, whereas attachment occurred in less than 7% of non-reactive cells (Table III). In *P. tetraurelia* the percentage of maximum attachment averaged 44% even when stocks hr^d of mating type VII and VIII were used. These are highly mating reactive mutants derived from stock 51 (Sonneborn, 1974). This low percentage of attachment probably reflects the presence of mating non-reactive cells in the sample because about 20% of cells used to test for attachment were found to be undergoing autogamy, and autogamous cells are known not to express mating reactivity (Metz, 1954).

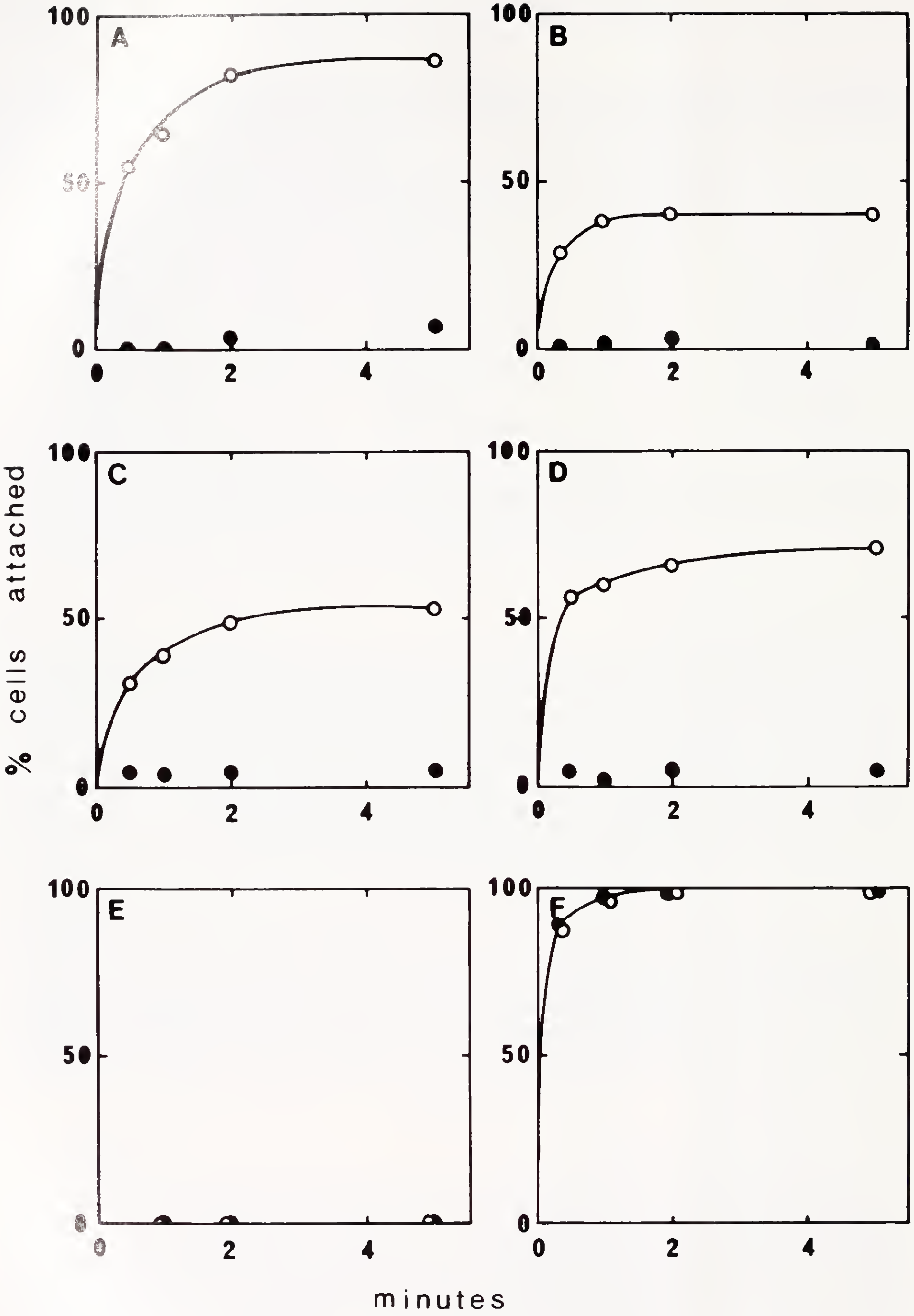

FIGURE 1. Typical adhesion kinetics of cells to the surfaces of polystyrene dishes in six species of *Paramecium*. Cells were adapted in glass tubes with a solution containing 1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1, for 10 min at 21°C. After adaptation, they were transferred into Petri dishes to test for adhesiveness. A; *P. caudatum:* B; *P. multimicronucleatum:* C; *P. tetraurelia:* D; *P. trichium:* E; *P. bursaria:* F; *P. duboscqui.* Open circles = stationary phase cells with mating reactivity. Closed circles = mating non-reactive cells in log phase of growth.

TABLE III

Attachment of cells to the surface of polystyrene dishes in P. tetraurelia *and* P. trichium, *syngen 1*

Stocks	Mating type	% Cells attached to the dishes	
		Non-reactive cells	Mating reactive cells
P. tetraurelia			
hr^d	VII	4 (1–8)	39 (32–46)
hr^d	VIII	3 (1–5)	45 (38–53)
d4-186	VII	5 (2–9)	44 (38–51)
d4-186	VIII	4 (2–7)	48 (42–54)
P. trichium			
So 1	I	3 (0.5–5)	64 (56–71)
Sw 1	I	7 (5–11)	52 (45–59)
So 2	II	4 (1–8)	65 (58–72)
Sw 2	II	5 (3–8)	60 (54–66)

In contrast to *P. caudatum,* micronuclei in *P. tetraurelia* normally do not lie close to the macronucleus, and the obvious micronuclear movement corresponding to the EMM in *P. caudatum* is not present in normal conjugation. No marked change in micronuclear size or behavior was observed even 2 h following attachment.

In mating reactive *P. trichium* cells, a maximum attachment of about 60% occurs within one minute following introduction of the cells to the polystyrene dishes (Fig. 1D). Attachment was rarely seen in non-reactive cells.

In stationary phase cells of *P. trichium,* the micronucleus is normally closely associated with the macronucleus as in *P. caudatum.* Nonetheless, no changes in micronuclear size or behavior were induced in cells even after more than 4 h of attachment.

P. bursaria

Mating reactivity of *P. bursaria* syngen 1 appears only during the daytime in stationary phase cells. Cells that are in log phase of growth or in the night phase fail to show mating reactivity (Wichterman, 1948, 1953; Ehret, 1953; Cohen, 1964). Among the 17 natural stocks tested, attachment was rarely seen in either mating reactive or non-reactive cells (stationary phase cells in the night) during the first 5 min following the addition of cells to the dishes (Table IV, Fig. 1E) In addition to natural stocks, three F1 hybrid clones between stocks 492 and T81C2 also did not attach to polystyrene (data not shown). Attachment was not seen in non-reactive cells of other states such as log phase in the daytime or at night. However, a marked change in swimming behavior occurred about 10 min after the transfer of cells into the dishes. Cells stopped swimming and remained near the bottom of the dish. By 30 or 40 min, more than 80% of cells showed cessation of swimming. Some cells remained immobile at the surface of the medium. Although the tracks of such resting cells can not be easily distinguished on the photographs from those attached to polystyrene surfaces, the resting cells did not show a clear attachment to polystyrene. They sometimes showed creeping, though very slowly, along polystyrene surfaces. Similar cessation of swimming was seen when cells were applied to glass dishes. Neither symbiotic *Chlorella*-containing stocks nor stocks deprived of their symbiotic green algae showed attachment (Table IV). This indicates that the algae did not inhibit attachment. Attachment was not seen at 30°C or even when the swimming velocity of cells was reduced to 45% of the normal velocity by increasing the external KCl concentration to 15 m*M*.

TABLE IV

Attachm[illegible]*ls to the surface of polystyrene dishes in* P. bursaria, *syngen 1*

			% Cells attached to the dishes	
Stocks	Mating type	Geographical origin	Non-reactive[a]	Mating reactive[b]
So 11	I	Nagoya, Japan	1.6	3.3
Ih 1*	I	Iwate, Japan	3.9	0
Ok 1	I	Aichi, Japan	0	0
So 13	II	Nagoya, Japan	2.8	0
Cs 2	II	Shanghai, China	2.3	0.8
Ok 4	II	Aichi, Japan	5.3	5.1
M 4	II	Miyagi, Japan	9.6	2.4
F 36	II	Nagoya, Japan	0	0
F 29	III	Nagoya, Japan	0	0
T 157	III	Ibaraki, Japan	5.3	5.1
So 5	III	Aichi, Japan	1.1	0
T 151*	IV	Ibaraki, Japan	0.9	1.7
T 316	IV	Ibaraki, Japan	0.9	1.5
Osk 3	IV	Osaka, Japan	2.7	0
Osk 4	IV	Osaka, Japan	1.6	2.1
Nn 7*	IV	Niigata, Japan	0	0
Nn 8	IV	Niigata, Japan	0	0

* Symbiotic *Chlorella*-free strain.

[a] Stationary phase cells at night.

[b] Stationary phase cells in the daytime.

However, if cells were treated with the hydrophobic reagents 0.5 m*M* benzylamine or phenethylamine for 10 min, more than 80% of the cells showed attachment within 2 min after pouring cells into the dishes and more than 90% of the cells were still attached as much as 5 h later. About 40% of cells treated with trypsin (50 μg/ml) for 30 min also showed attachment. However, this effect of trypsin was obtained only in mating reactive cells even though their mating reactivity was completely destroyed by the treatment. Trypsinization of non-reactive cells did not induce attachment.

In normal conjugation of the *P. bursaria* stocks used in this study, micronuclear movement corresponding to the EMM found in *P. caudatum* was not observed. Before conjugation, most micronuclei lie near the macronucleus. The micronucleus increases in size about 5 h after onset of the mating reaction. However, when mating reactive cells were induced to attach to dishes by pre-treatment with 0.5 m*M* benzylamine, no nuclear changes were evident up to 6 h following attachment.

P. duboscqui

Unlike the other 5 species of *Paramecium* used in this study all 15 stocks of *P. duboscqui* tested showed attachment even when cells were non-reactive for mating (Table V). When the attachment process was examined with an interference microscope, the attachment was shown to occur first at the tips of the antero-ventral cilia of both mating reactive and non-reactive cells. The attachment kinetics of these two types show that more than 90% of the cells adhered to polystyrene within the first minute following introduction of the cells to the dishes (Fig. 1F). Photos of cell tracks taken at 1 h intervals showed that almost all of the cells were still attached more than 6 h later, and such cells showed normal swimming behavior when transfered to glass

TABLE V

Attachment of cells to the surface of polystyrene dishes in P. duboscqui

Stocks	Mating type	% Cells attached to the dishes	
		Non-reactive	Mating reactive
1a	I	98	100
1b	I	96	99
11	I	98	95
12	I	97	98
15	I	97	97
22	I	97	95
23	I	99	96
24	II	96	98
25	II	88	94
26	I	95	77
31	I	88	98
32	I	97	96
34	I	95	93
37	I	86	91
310	I	80	82

Cells were washed and suspended in a solution containing 1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1, at 20°C. Ten min later, 5 ml of the cell suspension of each stock was introduced into polystyrene dishes.

Petri dishes with a micropipette. Strong affinity for polystyrene surfaces was also observed when cells were applied to Falcon 1001 or lids of Falcon 3002 dishes. These are less hydrophobic than Falcon 1007 dishes generally used in this study. More than 90% of cells showed attachment to both kinds of dishes regardless of mating reactivity.

No clear morphological or positional changes were observed in the micronucleus when cells were examined up to 5 h following attachment to polystyrene surfaces.

DISCUSSION

In this study, differences in adhesiveness of cells to polystyrene dishes were demonstrated among six species of *Paramecium.* These species can be classified into three groups based on their adhesiveness to polystyrene. Group I includes *P. caudatum, P. multimicronucleatum, P. tetraurelia,* and *P. trichium* which show mating reactive-dependent attachment. Group II is represented by *P. duboscqui* which shows mating reactive independent attachment. No attachment occurs in group III unless cells are pretreated with hydrophobic reagents as seen in *P. bursaria.* In group I, a considerable difference is found in the degree of adhesiveness among the four species. They can be arranged depending on the degree of attachment as follows; *P. caudatum* > *P. trichium* > *P. tetraurelia* $\simeq$ *P. multimicronucleatum* (Tables I–III, and Fig. 1). However, as mentioned in the Results, the *P. tetraurelia* cell population contained some autogamous cells. These may not attach to the dishes due to lack of mating reactivity. Consequently, the percentage of attachment in mating reactive cells may be underestimated.

P. bursaria cells tend to stop their movement 10 to 30 min after transfer into dishes. Does this cessation of swimming indicate attachment to polystyrene surfaces? Iwatsuki and Naitoh (1979) reported that *P. bursaria* syngen 1 cells show thigmotaxis

5 to 30 min after applying them to glass dishes. Cells that attach to polystyrene surfaces usually do so upon initial contact with the surface and a marked inhibition of ciliary movement is seen (Kitamura, 1982; Kitamura and Hiwatashi, 1984), while collision of cells with solid surfaces induce thigmotaxis (Iwatsuki and Naitoh, 1979). Additionally, microscopical observations of the cells with thigmotaxis revealed that most cells were creeping along the surface. Therefore, those two phenomena are clearly distinguishable from one another.

It is uncertain whether there is a correlation between attachment ability and classifications based upon cell shape as described by Woodruff (1921). All 'aurelia' group species used, *i.e., P. caudatum, P. multimicronucleatum,* and *P. tetraurelia,* belong to attachment group I. However, *P. trichium* which belongs to the 'bursaria' group (III) also showed mating reactive-dependent attachment (Table III). It should be noted that *P. duboscqui* whose body shape is of intermediate size (Chatton and Brachon, 1933; Jin *et al.,* 1981) has a different type of attachment from 'aurelia' and 'bursaria' groups.

Other ciliates also fall into the three attachment groups defined above. For example, *Tetrahymena thermophilia* (Wolfe and Colby, 1981; Kitamura, unpub. obs.), *Blepharisma japonicum* (Kitamura, unpub.), and *Pseudomicrothorax dubius* (Peck, pers. comm.) belong to group II and *Euplotes octocarinatus* and *Paraurostyla weissei* belong to group III (Kitamura, unpub.). Ciliates which show attachment only when they are not mating reactive have not yet been found.

Unlike in *P. caudatum,* attachment in *P. tetraurelia, P. trichium,* and *P. duboscqui* failed to provoke any obvious changes in the micronucleus such as EMM or an increase in its size. These results were predictable since the EMM of normal conjugation is observed only in *P. caudatum.* Other obvious micronuclear changes are not seen during the initial step of conjugation in species other than *P. caudatum.* In those species a direct contact of cell bodies of polystyrene might be necessary for nuclear activation.

Finally the observation of different adhesiveness to polystyrene surfaces among six species of *Paramecium* suggests differences in hydrophobicity of ciliary membrane surfaces among them. Our further studies are directed toward biochemically analysing the components of their ciliary membranes.

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A COMPARATIVE STUDY OF MELANIN-CONCENTRATING HORMONE (MCH) ACTION ON TELEOST MELANOPHORES

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ABSTRACT

The action of melanin-concentrating hormone (MCH) on melanophores was studied in 27 teleost species. MCH caused melanosome aggregation in all teleosts studied, including two siluroid catfish in which melanin-aggregating nerves are known to be cholinergic. In most fish, the minimal effective concentration of MCH was estimated to be 10^{-10} *M*, while in three swellfish examined it was higher than 10^{-8} *M*. The mode of action of the peptide was identical in either adrenergically or cholinergically innervated melanophores. It may act through specific receptors on the melanophore membrane. These results suggest that MCH may be a biologically active hormone common to teleosts.

INTRODUCTION

Enami (1955) observed pigment aggregation in catfish (*Parasilurus asotus*) melanophores after injecting crude extracts of the pituitary of the same species. He termed the effective principle "melanophore-concentrating hormone" or "MCH," and further indicated that the substance was neither a catecholamine nor an acetylcholine. Imai (1958) confirmed these results. Using the rainbow trout, *Salmo gairdneri,* Baker and Rance (1983) reported that "melanin-concentrating hormone" is a peptide with a molecular weight of less than 2000, and that most of its bioactivity occurs in the hypothalamus, favoring the hypothalamic origin of the hormone theory, which had originally been surmised by Enami (1955). Using immunohistochemical electron microscopy, the results of Naito *et al.* (1985) supported these findings.

Meanwhile, Kawauchi and his colleagues (1983) determined the primary structure of MCH peptide purified from chum salmon (*Oncorhynchus keta*) pituitary. Unrelated to any known hormonal peptide, it was found to be a novel hormone, consisting of 17 amino acids and a sulfhydryl link. Subsequently, the same molecular species of MCH was synthesized and its action to aggregate melanosomes (melanin-bearing dark organelles within melanophores) was confirmed in a few teleosts by Wilkes *et al.* (1984). The mechanism of MCH action now has been studied in detail in blue damselfish (*Chrysiptera cyanea*) melanophores (Oshima *et al.,* 1985).

Pigment aggregation in chromatophores is controlled primarily by sympathetic postganglionic fibers and peripheral transmission is adrenergic in many teleosts (Fujii, 1961; Fujii and Miyashita, 1975; *cf.* review by Fujii and Oshima, 1986). Recently, however, unusual melanophores have been reported in some species of siluroid catfish which receive cholinergic innervation (Fujii and Miyashita, 1976; Fujii *et al.,* 1982; Kasukawa *et al.,* 1986). Furthermore, melanophores which possess both alpha adrenoceptors and muscarinic cholinoceptors have been found in mailed cat-

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fish belonging to the genus *Corydoras* (Callichthyidae) (Kasukawa and Fujii, 1985; Kasukawa *et al.,* 1986). The action of the peptide on those cells as yet has not been studied. The present paper examines comparatively the effect of the hormone on melanophores of several teleost species in an attempt to determine whether MCH is a general color change hormone in Teleostei.

MATERIALS AND METHODS

The topmouth gudgeon (*Pseudorasbora parva*), rose bitterling (*Rhodeus ocellatus ocellatus*), and the top minnow (*Gambusia affinis*) were collected from Lake Inba, Chiba Prefecture. The marine catfish (*Plotosus lineatus*), rudder fish (*Girella punctata*), footballer (*Microcanthus strigatus*), five-banded damselfish (*Abudefduf vaigiensis*), dusky damsel (*A. notatus*), multicolorfin rainbowfish (*Halichoeres poecilopterus*), gluttonous goby (*Chasmichthys gulosus*), frogfish (*Istiblennius enosimae*), redfin velvetfish (*Hypodytes rubripinnis*), scribbled toby (*Canthigaster rivulata*), and the grass puffer (*Takifugu niphobles*) were collected near the Kominato Marine Biological Laboratory, Awa, Chiba Prefecture. Juveniles of the Japanese flounder (*Paralichthys olivaceus*) were supplied by the Chiba Prefectural Seawater Fisheries Experimental Station. Other species were obtained from local retail sources. All species are enumerated without omission later in the Results section. Freshwater and marine materials were maintained in freshwater and seawater aquaria, respectively. All materials were kept there for at least two days before use.

Split fin pieces from either the tail or dorsal fin of some species were prepared according to a method described previously (Fujii, 1959). As for the eel, siluroid catfish, and other scaleless species, small skin specimens from the dorsal trunk were prepared according to the method of Fujii and Miyashita (1976). In other teleosteans, scales were excised from the dorsal trunk. In the peppered catfish, *Corydoras paleatus,* melanophores on the inner surface of an isolated scale were observed (Kasukawa and Fujii, 1985). All the skin specimens were prepared in a physiological solution of the following composition (m*M*): NaCl 128, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.8, D-glucose 5.6, Tris-HCl buffer 5.0 (pH 7.2).

In some experiments, nervous elements around the chromatophores were stimulated chemically to liberate sympathetic pigment-aggregating transmitter (Fujii, 1959). For this purpose, a saline solution containing 50 m*M* K^+ in which the concentration of Na^+ was lowered to maintain constant tonicity was employed (Kasukawa and Fujii, 1984).

Other procedures and experimental arrangements were essentially the same as those described elsewhere (Fujii and Miyashita, 1975). To record the response of melanophores photoelectrically, however, an improved method was adopted (Oshima and Fujii, 1984).

The drugs used were norepinephrine hydrochloride (Sankyo, Tokyo), phentolamine mesylate (Ciba-Geigy, Basel), propranolol hydrochloride (Sigma Chemical, St. Louis), acetylcholine chloride (Daiichi Seiyaku, Tokyo), atropine sulfate (Tanabe Seiyaku, Tokyo), melatonin (Nakarai Chemical, Kyoto), and alpha melanophore-stimulating hormone (alpha-MSH, Sigma Chemical). Synthetic salmon melanin-concentrating hormone (MCH) was provided by Dr. Mac E. Hadley of the University of Arizona.

All experiments were performed at a room temperature between 20 and 26°C.

TABLE I

Teleostei materials employed in the present study to examine the action of MCH on melanophores, which were all proved sensitive to the peptide

ORDER Family	Zoological name	Common name
ANGUILLIFORMES		
Anguillidae	*Anguilla japonica*	Japanese eel
CYPRINIFORMES		
Cyprinidae	*Pseudorasbora parva*	topmouth gudgeon
	Rhodeus ocellatus ocellatus	rose bitterling
	Brachydanio rerio	zebra danio
Cobitidae	*Acanthophthalmus kuhlii*	coolie loach
SILURIFORMES		
Siluridae	*Parasilurus asotus**	Japanese common catfish
	*Kryptopterus bicirrhi**	translucent glass catfish
Ictaluridae	*Ictalurus punctatus*	channel catfish
Plotosidae	*Plotosus lineatus*	marine catfish
Callichthyidae	*Corydoras paleatus***	peppered catfish
CYPRINODONTIFORMES		
Oryziatidae	*Oryzias latipes*	medaka
Poecilidae	*Xiphophorus maculatus*	platyfish (blue variety)
	Gambusia affinis	top minnow
CHANNIFORMES		
Channidae	*Channa argus*	Northern snakehead
PERCIFORMES		
Girellidae	*Girella punctata*	rudder fish
Scorpididae	*Microcanthus strigatus*	footballer
Pomacentridae	*Abudefduf vaigiensis*	five-banded damselfish
	A. notatus	dusky damsel
Labridae	*Halichoeres poecilepterus*	multicolorfin rainbowfish
Gobiidae	*Rhinogobius brunneus*	common freshwater goby
	Chasmichthys gulosus	gluttonous goby
Blenniidae	*Istiblennius enosimae*	frogfish
SCORPAENIFORMES		
Congiopodidae	*Hypodytes rubripinnis*	redfin velvetfish
PLEURONECTIFORMES		
Paralichthyidae	*Paralichthys olivaceus*	Japanese flounder
TETRAODONTIFORMES		
Tetraodontidae	*Canthigaster rivulata*	scribbled toby
	Takifugu niphobles	grass puffer
	Tetradon fluviatilis	green puffer

* Species in which pigment aggregation in response to nervous stimulation is mediated by muscarinic cholinoceptors (Fujii and Miyashita, 1976; Fujii *et al.*, 1982).

** Species in which melanophores are under adrenergic nervous control, but which possess cholinoceptors of unknown significance (Kasukawa and Fujii, 1985).

RESULTS

Effects of MCH on adrenergically innervated melanophores

The effect of MCH on the melanophores of several fish species in which the pigment cells are adrenergically innervated was studied. These are listed in Table I.

Among these species, the following already have been shown to have adrenergically controlled melanophores: the medaka (Watanabe *et al.*, 1962), top minnow (Colley and Hunt, 1974), common freshwater goby (Naitoh *et al.*, 1985), gluttonous

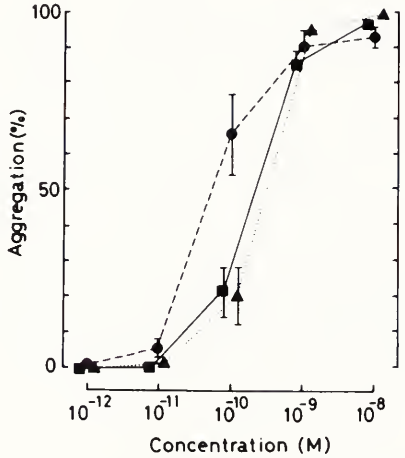

FIGURE 1. Relationships of the concentration of MCH to the magnitude of the pigment-aggregating response of melanophores of the translucent glass catfish, *Kryptopterus bicirrhi* (circles), the Japanese catfish, *Parasilurus asotus* (squares), and the zebra danio, *Brachydanio rerio* (triangles). MCH solutions of various strengths were applied for 7 min. The treatment was immediately followed by a 5-min application with 5×10^{-5} *M* norepinephrine or 10^{-5} *M* melatonin solution to bring about the full level of pigment aggregation in zebra danio or catfish melanophores, respectively. Abscissa: molar concentration of MCH (logarithmic scale). Ordinate: magnitude of response attained during MCH treatment in percentage. Each point is the mean of seven measurements on different animals. Vertical lines indicate SE.

goby (Fujii, 1961), and the Japanese flounder (Nagai *et al.*, 1985). These results were confirmed in the present study. In addition, our present pharmacological tests on other species listed above indicate that their pigment cells were likewise under the control of the adrenergic system. The exceptional cases in which melanophores are controlled cholinergically will be dealt with later.

In all of the aforementioned species, MCH induced a remarkable pigment aggregation in melanophores. After noting this, we made a quantitative measurement of one species, *i.e.*, the zebra danio, *Brachydanio rerio*. As shown in Figure 1, MCH acted on the effector cells dose-dependently, and the minimal effective concentration was estimated to be less than 10^{-10} *M*.

In many other species, too, the peptide was found to be effective at concentrations of not more than 10^{-10} *M*. Strangely, however, the melanophores of all three tetraodontid species examined were found to be less sensitive to MCH: discernible responses were only observable at above 10^{-8} *M*.

In melanophores of many of the above-mentioned species, the pineal hormone, melatonin, has been shown to be very effective in arousing pigment aggregation. Even after withdrawal of the hormone, a moderately aggregated state continued for a long period of time, as exhibited in Figure 2A, in which the response of melanophores of the topmouth gudgeon is recorded. When MCH was added immediately after the melatonin treatment, a further aggregation took place, as shown in Figure 2B. On the other hand, alpha-MSH, used as the control peptide produced a rapid dispersion of cellular inclusions (Fig. 2C). It was thus concluded that MCH has no ability to disperse melanosomes, but is definitely pigment aggregating.

It also should be noted here that the action of melatonin was rather differential among melanophores in many species as has already been reported in some species of fish (Reed, 1968; Fujii and Taguchi, 1969; *cf.*, Fujii and Oshima, 1986). An exam-

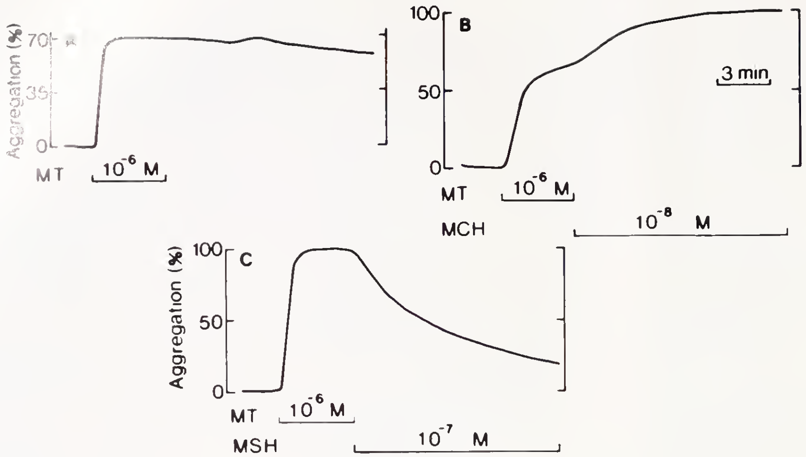

FIGURE 2. A set of recordings of the response of melanophores of the topmouth gudgeon, *Pseudorasbora parva,* indicating that MCH had no pigment-dispersing effect. The recordings were obtained on different scales but from the same fish. The magnitude of response is expressed as the percentage of the maximal level of pigment aggregation. In each recording, the scale was first treated with 10^{-6} *M* melatonin (MT) for 4 min. Then the perfusing medium was changed to physiological saline (A), 10^{-8} *M* MCH (B), or to 10^{-7} *M* MSH (C).

ple of this differential responsiveness to melatonin is exhibited in the melanophores of the topmouth gudgeon (Fig. 3B). On the other hand, the response to MCH proceeded quite simultaneously among the cells (Fig. 3C).

An alpha-adrenolytic agent, phentolamine, had no influence on the melanin-aggregating action of the peptide. As an example of the records, the response of the topmouth gudgeon is exhibited in Figure 4. The melanin-aggregating action of K^+, however, was easily inhibited by phentolamine. This ion has been known to act on chromatic neural elements around the pigment cells to release neurotransmitter (Fujii, 1959). It was concluded, therefore, that MCH acts directly on the target cells and not through activities of the neural elements. Although not shown in the figure, it was further shown that a beta adrenergic blocker, propranolol, had no effect on the action of MCH. These results further indicate that MCH acts independently of alpha and beta adrenoceptors.

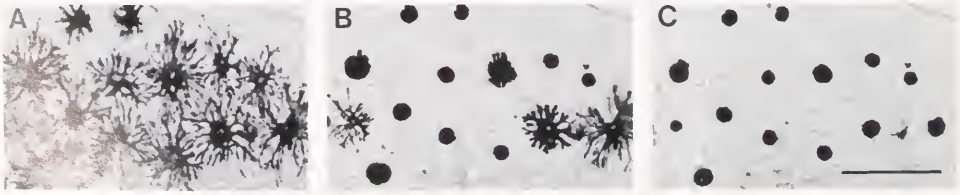

FIGURE 3. Photomicrographs of part of a scale isolated from dorsal part of the topmouth gudgeon, showing the effects of melatonin and MCH. Scale bar indicates 150 μm. A: Equilibrated in physiological saline. Melanosomes in melanophores are in a fully dispersed state. B: 10 min after the application of 5×10^{-7} *M* melatonin. The pigmentary organelles in some melanophores remain in a dispersed state, while in others they have become completely aggregated in the perikarya. C: 10 min after the application of 10^{-7} *M* MCH. Melanosomes in all melanophores are completely aggregated.

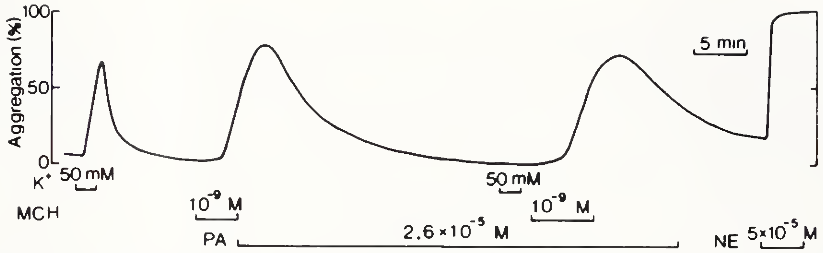

FIGURE 4. Typical recording showing that the alpha adrenolytic agent, phentolamine (PA), did not block the melanin-aggregating effect of MCH in the topmouth gudgeon, *Pseudorasbora parva.* Ordinate: magnitude of response as percentage of the maximal pigment aggregation attained finally by 5×10^{-5} *M* norepinephrine (NE). 50 m*M* K^+ Ringer (K^+) was used to stimulate adrenergic neural elements around the melanophores, and the action was shown to be antagonized by phentolamine (PA).

The melanosome-aggregating response to MCH proceeded quite normally even in the absence of Ca ions. This is in marked contrast to the alpha-MSH action on teleostean chromatophores, in which Ca^{2+} was definitely required (Fujii and Miyashita, 1980; Iga and Takabatake, 1982; Oshima and Fujii, 1985).

Melanophores of the European eel, *Anguilla anguilla,* already have been recognized to be influenced only weakly by the nervous system (Neill, 1940; Gilham and Baker, 1984). Recently, Baker and Rance (1983), however, indicated that their melanophores were responsive to MCH. Although the species used was different, melanophores of the Japanese eel (*A. japonica*) were found in this study to respond quite well to MCH by pigment aggregation.

Effect of MCH on cholinergically innervated melanophores

MCH effectively induced the aggregation of melanophore pigment in the translucent glass catfish, *Kryptopterus bicirrhi,* and the Japanese common siluroid, *Parasilurus asotus,* as seen in Figures 5 and 6, respectively. Both fish belong to the family Siluridae (Siluriformes), and recently have been shown to have melanophores cholinergically controlled (Fujii and Miyashita, 1976; Fujii *et al.,* 1982; *cf.* also Table I). The action of MCH was not inhibited by atropine, a muscarinic cholinolytic, but the pigment-aggregating effect of K^+ was completely blocked by the same drug (Fig. 5). In addition, divalent cation-withdrawal exhibited no influence on the action of MCH (Fig. 6).

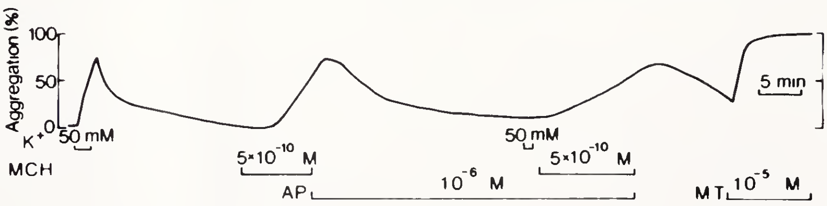

FIGURE 5. Typical recording showing the ineffectiveness of the muscarinic cholinolytic agent, atropine (AP), in blocking the pigment-aggregating action of MCH on melanophores of the translucent glass catfish, *Krytopterus bicirrhi.* Melatonin (MT; 10^{-5} *M*) was applied finally to bring about the full level of melanosome aggregation. 50 m*M* K^+ Ringer (K^+) was used for neural stimulation.

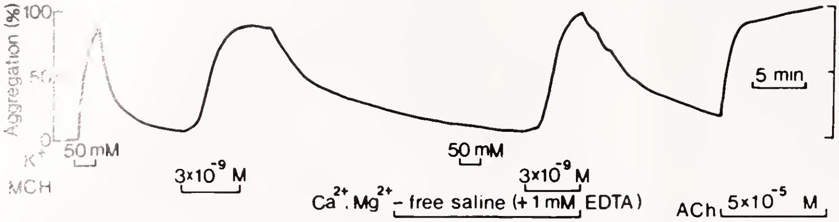

FIGURE 6. Typical recording of the response of Japanese common catfish (*Parasilurus asotus*) melanophores to MCH, showing that the pigment-aggregating effect of the peptide was still remarkable in Ca^{2+}-free saline, in which the release of peripheral stores of neurotransmitter (ACh) was inhibited. 50 m*M* K^+ Ringer (K^+) was used for neural stimulation. Finally, 5×10^{-5} *M* acetylcholine (ACh) was applied to induce the full aggregation of melanosomes.

The relationship between the strength of MCH and the magnitude of the target cell response in the glass catfish and the common siluroid was revealed to be dose-dependent, as shown in Figure 1. In both species, the threshold concentration was estimated to be not more than 10^{-10} *M*, and a response larger than 80% of the maximal one was induceable at a concentration of 10^{-9} *M*.

Effects of MCH on melanophores possessing both alpha-adrenoceptors and cholinoceptors

Melanophores of the mailed catfish of the genus *Corydoras* (Callichthyidae, Siluriformes) possess melanosome-aggregating cholinoceptors of the muscarinic type, notwithstanding the fact that they are also under the control of adrenergic fibers (Kasukawa and Fujii, 1985; Kasukawa *et al.,* 1986; *cf.* also Table I). Employing a representative species from this genus, *i.e.,* the peppered catfish (*C. paleatus*), we found that MCH had the same potent action to aggregate melanophore inclusions as it did in other teleosteans. Neither adrenolytic nor muscarinic cholinolytic agents interfered with the MCH effect. Furthermore, Ca^{2+}-withdrawal did not affect the responsiveness to the hormone.

DISCUSSION

Using a number of teleost species from a variety of families and orders, we have demonstrated in the present study that MCH is very effective in bringing about a notable pigment aggregation not only in adrenergically but also in cholinergically innervated melanophores, and further that the mode of action of the peptide is the very same among those pigment cells. Its minimum effective concentration was estimated to be less than 10^{-10} *M* in most teleosts, with the exception of three swellfish: the scribbled toby, the grass puffer, and the green puffer.

Recent quantitative studies on the effects of other pigment-motor hormonal substances, *i.e.,* MSH (Fujii and Miyashita, 1982) and melatonin (Fujii and Miyashita, 1978), showed that the threshold concentration was about 10^{-10} *M*. Our present results on MCH agree well with those data. In the above-mentioned swellfish, however, the threshold concentration of MCH for inducing a discernible melanin aggregation was estimated to have an exceptionally high value of about 10^{-8} *M*. Further comparative studies among species of Tetraodontidae are thus required to give an adequate explanation for such lower sensitivity to the hormone.

Using purified or synthetic chum salmon MCH, Kawauchi *et al.* (1983), Wilkes *et al.* (1984), and Oshima *et al.* (1985) have already demonstrated MCH's pigment-aggregating action on the adrenergically innervated melanophores of a number of teleost species. These species are the rainbow trout, *Salmo gairdneri* (Salmonidae, Salmoniformes), the carp, *Cyprinus carpio* (Cyprinidae, Cypriniformes), the crucian carp, *Carassius auratus* (Cyprinidae, Cypriniformes), the fathead minnow, *Pimephales promelas* (Cyprinidae, Cypriniformes), the guppy, *Lebistes reticulatus* (Poeciliidae, Cyprinodontiformes), the swordtail, *Xiphophorus helleri* (Poeciliidae, Cyprinodontiformes), the black rockfish, *Sebastes schlegeli* (Scorpaenidae, Scorpaeniformes), the fat greenling, *Hexagrommos otakii* (Hexagrammidae, Scorpaeniformes), the tilapia, *Sarotherodon mossambicus* (Cichlidae, Perciformes), the blue damselfish, *Chrysiptera cyanea* (Pomacentridae, Perciformes), and the clingfish, *Gobiesox pinniger* (Gobiesocidae, Gobiesociformes). Incidentally, Wilkes *et al.* (1984) referred to the last fish as *Gobeisox,* giving neither the common name nor any taxonomical keys. Apparently the genus name given involved a clerical error. It should have read *Gobiesox,* the genus, we confirmed, comprising the species *G. pinniger* which is indigenous to the United States. In addition, Baker and her colleagues (Baker and Rance, 1983; Gilham and Baker, 1984) have also shown that partially purified MCH of trout (*Salmo gairdneri*) origin caused pigment aggregation in melanophores of the European eel, *Anguilla anguilla.*

In addition to the above-mentioned species, we have surveyed 27 other species of fish in this study, and found that each species' melanophores were reactive to MCH by pigment aggregation. To date, we have not encountered any fish in which the melanophores have been unresponsive to this hormone. From these results it may be deduced that MCH is a physiologically functioning hormone throughout the teleost class. As suggested by some workers (Enami, 1955; Baker and Rance, 1983; Naito *et al.,* 1985), the peptide, synthesized in the hypothalamus, may be transferred to the pituitary, wherefrom it is secreted as a hormone to blanch the integument in most teleosts.

The mode of action of MCH on cholinergically controlled melanophores has been studied for the first time in this work. Pharmacological analyses clearly indicate that the melanosome-aggregating action of MCH is not inhibited by a muscarinic cholinolytic agent, atropine, which normally interferes with peripheral transmission to the effector cells.

It is now known that Ca^{2+} is required for the release of neurotransmitters from chromatic nerves of either adrenergic (Fujii and Novales, 1972; Takabatake and Iga, 1982) or cholinergic type (Kasukawa and Fujii, 1985). Therefore, we have tried to test the influence of MCH in the absence of this ion. The result was that MCH exhibited its potent action irrespective of the presence of Ca^{2+}. Incidentally, we have lately reached the same conclusion on adrenergically controlled melanophores (Oshima *et al.,* 1985). In reconfirming that conclusion, we found that in cholinergically innervated melanophores the action of MCH is mediated through the specific receptors for MCH.

During the same period of time, it was noted that the differential responsiveness to melatonin seen among the melanophores of individual animals or on isolated pieces of skin may be responsible for the formation of the characteristic color pattern seen in the pencil fish *Nannostomus* (Reed, 1968), and possibly in some other teleosts (Fujii and Taguchi, 1969; Fujii and Miyashita, 1978; *cf.* also review by Fujii and Oshima, 1986). During the present investigation, we also found that the responsiveness to melatonin was actually very differential among melanophores distributing, for instance, over an isolated scale of the topmouth gudgeon. In contrast, MCH induced much more even responses among cells in most fish species. Therefore, we assume that MCH serves as a color change hormone whose action counteracts that of alpha-MSH. In preliminary experiments on the zebra danio, topmouth gudgeon, and the glass catfish, we examined the response of melanophores to solutions containing both alpha-MSH and MCH. When the strengths of both hormones were approximately equal, a moderately aggregated state of pigment could be maintained in the melanophores. It is still uncertain, however, to just what extent MCH takes part in actual physiological color changes in teleosts. For a full understanding of the role of the peptide, quantitative analyses, such as correlated measurement by radioimmunoassay of plasma MCH levels and reliable assessment of integumental chromatic reactions *in vivo,* should be performed.

The individual membranes surrounding teleost melanophores are endowed with a number of ligand receptor species which mediate pigment movements within the cell (Fujii and Oshima, 1986). They include at least those for MSH (Fujii and Miyashita, 1982), melatonin (Fujii and Miyashita, 1978), alpha- (Fujii and Miyashita, 1975), and beta-adrenergic amines (Miyashita and Fujii, 1975), and for adenosine (Miyashita *et al.,* 1984). The receptors for MCH now may be added to this list. Although not all of the above-mentioned receptors are always present in a single melanophore of a certain species of animal, very frequently many of them do exist, each carrying out its individual function (Fujii and Oshima, 1986). We know of no other instance among effector cells in which so many kinds of receptors exist and function to regulate the cells' motile responses. Representing a remarkable instance of possession of so many kinds of receptors, therefore, the fish melanophore may prove useful in studying the mechanisms by which many or large numbers of input signals are integrated into a simple vectoral movement of pigmentary organelles.

That so many receptor sorts are at work seems to signify the necessity for subtle and delicate regulation of the chromatophores for animals living in their habitat. If we remind ourselves of the fact that the color changes and color patterns displayed by animals are very important in their strategies for survival, the situation can even more easily be understood.

We hope that our present comparative studies at the cellular level will be of some use not only for further understanding of the cellular mechanism of hormonal action, but also for comprehension of the above-mentioned organismal phenomena of both ethological and ecological interests.

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LARVAL RELEASE IN RESPONSE TO A LIGHT SIGNAL BY THE INTERTIDAL SPONGE *HALICHONDRIA PANICEA*

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ABSTRACT

The intertidal sponge, *Halichondria panicea,* regularly begins releasing larvae shortly after dawn, and ejects most of them during morning hours under a natural light-dark (LD) cycle. Its diurnal periodicity was confirmed under an artificial LD 12:12h cycle. In search of a trigger that stimulates the sponge colonies to release larvae, the colonies were subjected to experimentally modified LD cycles. Under continuous darkness, only a single release peak was observed about fifteen hours after the beginning of darkness. Further, the colonies invariably released larvae about fifteen hours after the change from light to dark on the preceding day under all illumination regimes examined. The timing of their larval release was independent of both the tidal cycle and the daily cycle of seawater temperature. These results indicate that the trigger is a light signal: the onset of darkness (not onset of light) of the preceding day under natural illumination. Subsequent to this stimulus, *H. panicea* needs a period of fifteen hours before release actually occurs. This light-controlled larval release probably has ecological significance for habitat selection by intertidal sponges.

INTRODUCTION

Most intertidal sponges are viviparous (Hyman, 1940; Bergquist *et al.,* 1970). In these sponges, fertilized eggs develop into swimming larvae within the mesohyle, and then leave the parent sponge through the excurrent canals and oscula (Levi, 1956; Fell, 1969; Fell and Jacob, 1979). The ubiquity of viviparity among intertidal sponges suggests some ecological significance for their habitat selection. However, little is known about the larval release mechanism.

About thirty years ago, Levi (1951, 1956) showed that larval release occurs shortly after sunrise in several sponge species. Another species, however, released larvae throughout the day following their collection (Levi, 1956). According to incidental observations, several sponges extruded larvae for several hours after collection (Wilson, 1894; Ali, 1956; Fell, 1967, 1969; Fell and Jacob, 1979). In such instances, the release may be induced artificially by shock or confinement.

Halichondria panicea released the majority of its larvae during morning hours under natural illumination in our laboratory. Using experimental light regimes, the study described in the present paper demonstrates clearly the role of light in inducing larval release in *H. panicea.* The results facilitate a discussion of habitat selection by sponges from an ecological point of view.

MATERIALS AND METHODS

All *Halichondria panicea* colonies were collected from the shallow waters near the Asamushi Marine Biological Laboratory in Japan (40° 67′N: 140° 52′E). The greenish

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colonies, which are several centimeters thick, usually encrust rocks which are both exposed to the sun and washed by waves. Because these colonies attach broadly and firmly to the rocks, a sharp knife-blade was used carefully to free the colonies from the substrate, so as to minimize any damage. The freed sponges were transferred under water to individual water-tight containers and immediately brought to the laboratory. There they were placed quickly in running seawater. The sponge colonies treated in this manner appeared healthy and showed little degeneration during the laboratory investigations.

Early in the morning following collection, the sponge colonies were placed individually in still seawater. After one hour, those releasing a large number of larvae were selected for study. Only a small proportion of the collected colonies (about 20%) released many larvae in the laboratory.

For the study of larval release under various illumination schedules, a photographic developing tank was used as a light-shielding container for each sponge colony. The colony was fastened inside the tank and continuously supplied with fresh, clean running seawater while it was illuminated or completely shielded from light. The colonies were illuminated by a fluorescent light (40W × 2) mounted on the ceiling of the laboratory. The released larvae were washed by the outflow into a larva collector. The larva collector is a plastic vessel with a piece of nylon mesh (NXX 13, 94 μm) applied to a pore (about 3 × 5 cm) on one side of the vessel. The collected larvae were counted regularly.

RESULTS

Halichondria panicea colonies released larvae in August and September. Similarly, several other sponges release larvae in the later period of their reproductive season (Simpson, 1968; Chen, 1976; Fell and Jacob, 1979; Ayling, 1980). The larvae of this species are pale yellow and their size is about 400 × 250 μm. They are thickly covered with cilia except for a posterior pole which is bare. The bare pole is encircled by a band of long cilia. These parenchymula larvae swim rapidly with constant rotation as soon as ejected from the osculum.

In the laboratory early the next morning after collection, I found several *H. panicea* colonies releasing many larvae. Thus larval release was first studied under natural illumination. Usually, larval release began within one hour after dawn (about 5:30) and the majority of larvae were released before noon. A small number of larvae were released in the afternoon and almost none were released after dusk (about 18:30) until the next morning. This concentrated morning release was repeated during successive days.

This diurnal periodicity was confirmed under the artificial light-dark cycle (LD 12:12h, light period 6:00 to 18:00). A typical example for 24 hours is shown in Figure 1, since all the colonies examined released larvae in an essentially similar pattern. Under this LD cycle, the sponge colony began releasing larvae shortly after the beginning of the light period; release regularly peaked after a few hours (about 9:00). Most larvae were released during the first half of the light period (6:00–12:00) with fewer released in the latter half (12:00–18:00). Almost none were released in the dark period (18:00–6:00). This periodical release continued for more than a week (Fig. 2).

During these experiments, the seawater temperature changed diurnally (Fig. 1). Usually the temperature was high in the afternoon, low at night, and its difference within a day was about two degrees.

To investigate the mechanism that triggers the diurnal periodicity of larval release, the sponge colony was subjected to experimentally modified LD schedules. Figure 3

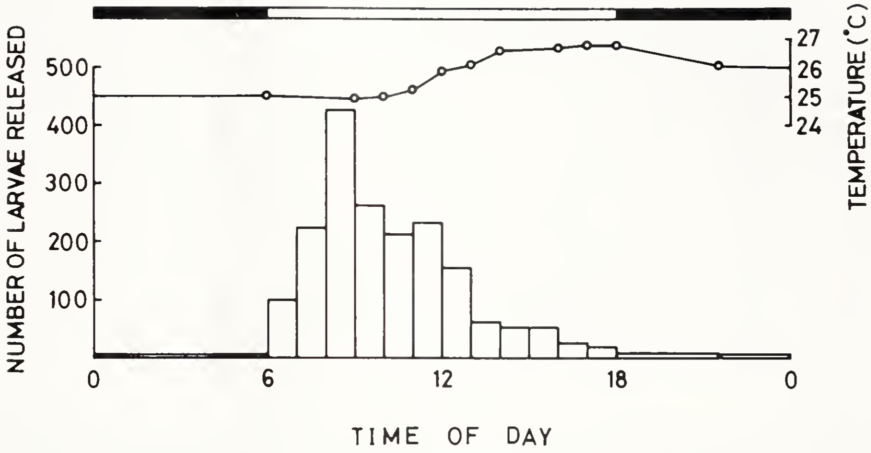

FIGURE 1. Typical pattern of larval release by *Halichondria panicea* during 24 hours under a LD 12:12h cycle. Included is the change in seawater temperature on that day. At the top, the illumination schedule is shown. The blackened bar indicates the dark period and the white portion, the light period.

shows the effect of continuous darkness on larval release. Here a sponge colony that had been under the LD 12:12h cycle was subsequently kept in the dark for 54 hours. Under continuous darkness, only a single peak of release occurred and no second peak was observed as long as darkness continued. Characteristically, the release peaked about 15 hours after the beginning of continuous darkness; this time corresponds exactly to when a peak would occur if the LD 12:12h cycle had been continued. This result shows that, first, even in the dark the sponge colony is initially able to release larvae and light is not necessary. Second, the release of larvae is not controlled by the circadian rhythm. Third, the stimulus that induces release is not the dawning light of that morning. Thus it is reasonable to conclude that the illumination on the preceding day determines the time of release.

Figure 4 shows larval release when the timing of the LD cycle on the preceding

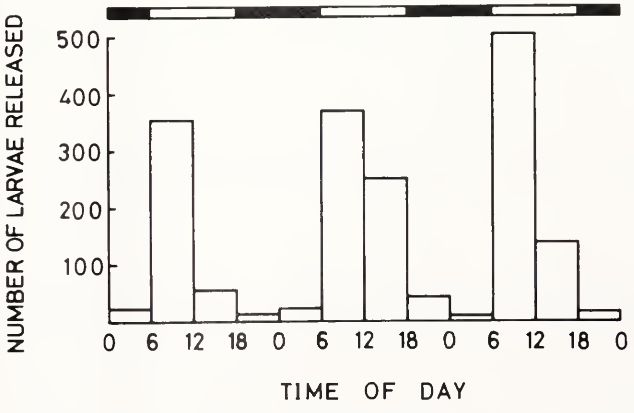

FIGURE 2. Diurnal periodicity of larval release by *Halichondria panicea* during three successive days under the LD 12:12h cycle. A release peak appeared regularly each day at about 9:00.

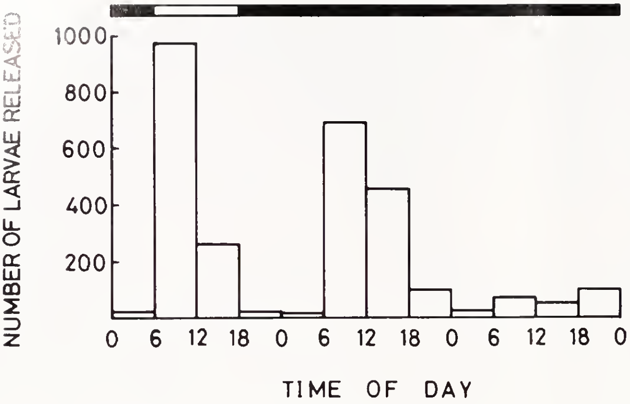

FIGURE 3. Larval release under continuous darkness. On the first day, the colony was illuminated (6:00–18:00) as in the LD 12:12h cycle then subsequently darkened for 54 hours. On the second day although it was dark, it released larvae at the same time as if still under the LD 12:12h cycle. On the third day, however, no concentrated release was observed.

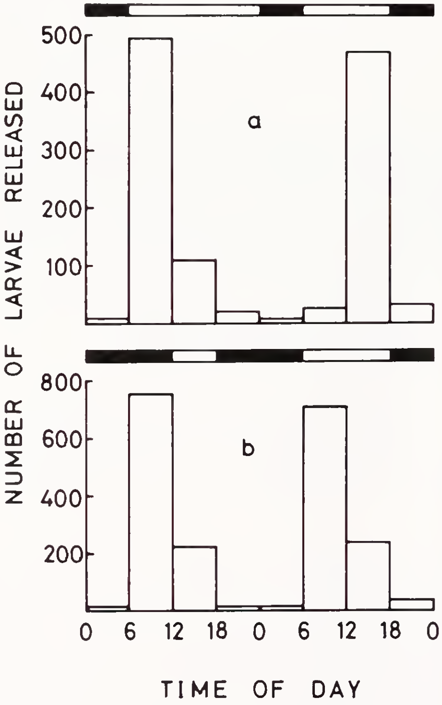

FIGURE 4. Larval release under modified light-dark cycles. (a) Delay in the onset of the dark period for six hours resulted in a delay of release for six hours on the next day. (b) Six-hour delay in the onset of the light period, however, did not bring about any delay in the next day's release.

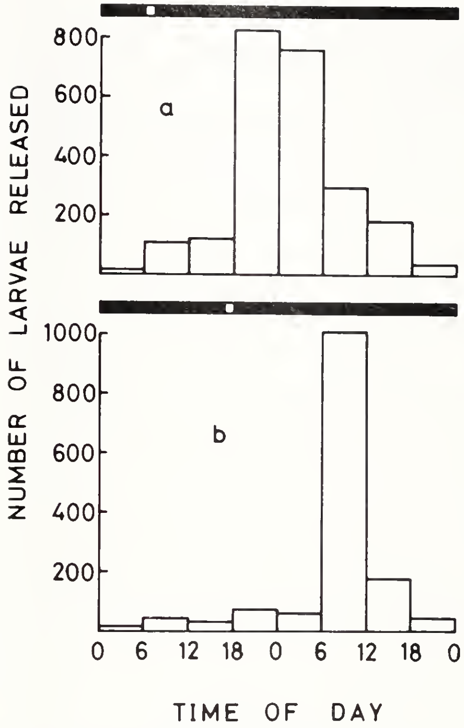

FIGURE 5. Larval release induced by illumination for one hour. (a) One hour of illumination in the morning (6:00–7:00) brought about a release peak at midnight. (b) One hour of illumination in the evening (17:00–18:00) brought about a release peak in the next morning. In both cases, the peaks appeared about fifteen hours after illumination.

day was modified. Figure 4a shows that a six-hour delay in the onset of the dark period on the preceding day resulted in delay of larval release for six hours. On the other hand, a six-hour delay in the onset of the light period had no effect (Fig. 4b). Thus, it is highly probable that the timing of larval release is determined by the change from light to dark (L to D) in the LD cycle on the preceding day.

Results presented in Figure 5 verify the above supposition. Preliminary experiments showed that illumination for one hour is sufficient to induce dark-adapted sponges to release larvae. If the trigger is really the L to D change in the preceding LD cycle, larvae should be released about 15 hours (18:00–9:00 in LD 12:12h cycle) after the one hour illumination. Figure 5 clearly indicates that this is the case because the peaks appeared after about 15 hours whether the sponge colonies were illuminated at dawn (6:00–7:00) or at dusk (17:00–18:00).

Table I summarizes the results graphed in Figures 2 to 5. Under the various LD regimes, the duration between the time of L to D change in a preceding LD cycle and the peak of larval release is always approximately fifteen hours. On the other hand, D to L changes of that morning or on the preceding day had no constant temporal relationship to the releases. These results indicate that the darkening at dusk is the true stimulus under natural illumination and that a delay of fifteen hours is necessary before larval release can occur. Furthermore, the length of the light periods (1–18 h) is apparently without effect under these experimental conditions.

TABLE I

Temporal relationship between light signals and larval release in Halichondria panicea *under various LD regimes*

D to L on that day*	L to D on the preceding day*	D to L on the preceding day*	Reference
3	15	27	Fig. 2
—	15	27	Fig. 3**
9	15	33	Fig. 4a**
3	15	21	Fig. 4b**
—	14	15	Fig. 5a
—	15	16	Fig. 5b

* Approximate durations (hours) between the time of light onset (D to L) or dark onset (L to D) and the peak of larval release.

** Peak of release on the second day.

DISCUSSION

Under natural illumination in the laboratory, *Halichondria panicea* colonies release the parenchymula larvae with diurnal periodicity: they release the majority of larvae during morning hours. *Haliclona permolis* also releases larvae in the morning (Amano, unpub. data). Similarly, Levi (1951, 1956) reported the release of larvae a short time after sunrise in *Oscarella lobularis, Hymeniacidon sanguinea,* and *Halisarca metschnikovi.* These five species represent most viviparous orders of the class Demospongiae. So although additional studies obviously are necessary, the concentrated release of larvae in the morning may be a common phenomenon among the Demospongiae.

H. panicea colonies were subjected to artificial light regimes in a search of an after-dawn larval release trigger. Results from these studies clearly indicate that the larval release of *H. panicea* is triggered by a light signal: the onset of darkness on the preceding day under natural illumination. Unfortunately, it is as yet unknown whether this light-controlled release is common among other sponges. Many colonial ascidians also release their tadpole larvae during morning hours under natural illumination. The release is also controlled by a light signal; however, it is not the onset of dark but the onset of light that triggers the release (Watanabe and Lambert, 1973). Why do these triggers differ while bringing about similar release patterns? The two patterns may have been acquired independently as a result of convergence in the remotely related phyla.

No environmental elements other than light exhibited any detectable influence upon the timing of larval release by *H. panicea.* It is independent of the daily cycle of seawater temperature and of the tidal cycle as well. Furthermore, it is unlikely that timing is controlled by the circadian rhythm since only a single peak of release was observed during continuous darkness.

Intertidal sponges may remain healthy for some time in the laboratory if maintained in suitable conditions (Fell, 1967). Sponge colonies must be collected carefully so as to minimize deleterious effects and constantly supplied with fresh, clean running seawater during the experiments. Colonies treated in this manner always reacted to the light signals for more than two weeks. Therefore, although results of field studies are not available for comparison, it is reasonable to consider that the larval releases observed in this study are not caused by uncontrollable artifacts (Wilson, 1894; Ali, 1956; Fell, 1967, 1969; Fell and Jacob, 1979) but reflect the release in their natural habitat.

Why do the sponges release larvae during the morning hours? As noted before, many colonial ascidians release larvae primarily in the morning (Watanabe and Lambert, 1973). Symbionts of algal-ascidians, however, release at midday (Olson, 1983). The behavior and settlement of these tadpole larvae are closely attuned to light conditions, with the larvae settling in shaded habitats (Millar, 1971). In the corals, planula larvae are positively phototactic upon release but later reverse this and become attracted to dark surfaces (Harrigan, 1972; Lewis, 1974). Because their free-swimming periods are relatively short (several hours in most cases), if released in the morning, the larvae should have a maximum period for seeking a suitable habitat during the day of release. The larvae of *H. panicea* swim rapidly for two days after release and are positively phototactic during this swimming phase. Several hours before settlement, however, their behavior changes; they creep around the substratum and are indifferent to light (Amano, unpub. obs.). On the other hand, adult sponges of this species usually inhabit the surfaces of rocks relatively exposed to the sun. Although larvae of many sponges can respond to light and gravity they do not have consistent behavior patterns (Warburton, 1966; Bergquist *et al.,* 1970; Fell, 1974). The duration of their free-swimming periods also varies greatly among species, from a few hours to twenty days in the laboratory. Thus in *H. panicea* and other species as well, it has not been possible to clearly elucidate the relationship between larval behavior and the habitats of the adult sponges. The sponge larvae may behave somewhat differently in their natural environments (Fell, 1974).

In the class Demospongiae, most sponges of the subclass Ceractinomorpha are viviparous. On the other hand, a larger part of other subclass Tetractinomorpha is oviparous (Levi, 1956, 1957; Bobojevic, 1966; Van de Vyver and Willenz, 1975; Reiswig, 1976; Watanabe, 1978). Marine sponges of the former subclass are common in the intertidal zone of temperate and tropical regions. In the intertidal zone habitat selection seems much more critical for free-swimming sponge larvae because the environmental conditions—light, seawater temperature, current, etc.—usually range more widely than on deeper bottoms (Meadows and Campbell, 1972). Embryos of viviparous sponges develop while being protected within the mesohyle for several weeks or months before they become swimming larvae. If eggs were released from an intertidal sponge, they might be carried far away from the intertidal region during the embryonic development thereby losing the chance to settle in a suitable habitat. Larvae released from intertidal sponges can swim actively, respond to environmental stimuli, and settle in a suitable habitat in a relatively short but critical free-swimming period. Therefore I suggest that viviparity is advantageous for the adaptation of sponges to intertidal existence.

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VARIATION OF CHELIPED ALLOMETRY IN A HERMIT CRAB: THE ROLE OF INTRODUCED PERIWINKLE SHELLS

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ABSTRACT

From Connecticut to Massachusetts, *Pagurus longicarpus* hermit crabs commonly inhabit shells of the introduced periwinkle snail, *Littorina littorea.* South of Connecticut, these periwinkles are rare and thus their shells are seldom inhabited. For male *P. longicarpus* along the Atlantic coast, use of periwinkle shells does not correlate with geographic differences in carapace length, but does correlate with geographic differences in cheliped allometry. Further, geographic patterns of shell use may indicate that periwinkle shells increased the shells available to *P. longicarpus* in New England. Such an increase in shell availability is associated with reduced relative cheliped growth in laboratory populations and may be responsible for the correlation between use of *L. littorea* shells and reduced right cheliped allometry in field populations. Museum specimens of male *P. longicarpus* from pre-*L. littorea* Connecticut also show significantly faster relative growth of the right cheliped than modern crabs.

INTRODUCTION

Empty snail shells are a critical resource for shell-living hermit crabs (Vance, 1972a; Fotheringham, 1976, Abrams, 1980; Bertness, 1981a), and hermit crab species and their inhabited shell species show a close evolutionary relationship (Jackson, 1913; Reese, 1969; Bertness, 1981b, 1982). Consequently, shells of introduced snails may affect hermit crabs dramatically (Drapkin, 1963). The European periwinkle, *Littorina littorea,* has become common along the northeast coast of North America in the last 150 years (Carlton *et al.,* 1982; Vermeij, 1982; Brenchley and Carlton, 1983; Bertness, 1984). Shells of this periwinkle are inhabited by the hermit crab *Pagurus longicarpus* in the northern part of its geographic range (see Fig. 1), and laboratory experiments suggest that these shells may have contributed to geographic variation in the morphology of *P. longicarpus* (Blackstone, 1985).

In this paper, the effects of *L. littorea* on *P. longicarpus* are elucidated by: (1) collecting data on geographic variation in carapace length, right cheliped allometry, and shell use of *P. longicarpus,* (2) correlating the morphological variation to the use of *L. littorea* shells, and (3) investigating observed associations further with modern estimations of shell availability and historical data on morphology. This study focuses on male *P. longicarpus* exclusively, since female *P. longicarpus* show less geographic variation than males and inhabit *L. littorea* shells less frequently (Blackstone, 1985).

MATERIALS AND METHODS

Geographic sampling

Collecting methods were constrained by: (1) the mobility of *P. longicarpus* (*e.g.,* Rittschof, 1980; Wilber and Herrnkind, 1982; pers. obs.), which makes quadrat sam-

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pling impractical (quadrat size should encompass an average individual's territory, Southwood, 1978), and (2) the estuarine habitat of these crabs, which makes subtidal sampling with SCUBA difficult because of motorboat traffic, strong tidal currents, and high sediment loads (thus low visibility). At each site, samples were collected at low tide by areal search in the shallow subtidal, parallel to the waterline, for approximately 1 h. The smallest possible area (range 5–50 m^2) that would yield a suitable sample size (about 100 crabs) was carefully searched. The size of the sample area varied inversely with the density of the crabs.

The effectiveness of areal search as a collecting method has recently been questioned (Gilchrist and Abele, 1984). Working with four hermit crab species in the Gulf of Mexico, Gilchrist and Abele (1984) compare the population parameters obtained by different sampling methods. For *P. longicarpus,* their predation site method, employing baiting, and their transect method, employing areal search, produce samples which do not differ significantly in crab size (Fig. 1 in Gilchrist and Abele, 1984). However, these samples do differ in the degree of shell damage; *P. longicarpus* collected at simulated predation sites more frequently occupy damaged shells (Fig. 2 in Gilchrist and Abele, 1984). Because shells are usually available at predation sites, their baiting method could be attracting a biased subsample of *P. longicarpus,* comprising those individuals most highly motivated to obtain a new shell. Further, the baiting method attracts few hermit crabs off the New England coast (M. A. Shenk, pers. comm.) and thus would be inappropriate for geographic comparisons. Gilchrist and Abele (1984) recommend employing a combination of the two techniques, but for these reasons the areal search method was considered the most appropriate for this study.

The sites, dates, sizes, and regional groupings of the samples collected in 1983–1985 are shown in Table I; this study also used samples from 1980–1982 (see Blackstone, 1985). The 60-day planktonic larvae of *Pagurus* hermit crabs (Nyblade, 1974) determined the regional assignments; sites which could easily exchange larvae were grouped in the same region (see Fig. 1). Further, the high vagility of this species determined the method of statistical analysis; with high rates of migration, annual samples at the same site can be considered independent and can vary as much as simultaneous samples at different sites. Each sample was treated as a replicate sample of the *P. longicarpus* of that region. Within-region spatial and temporal variation thus is considered part of the error variation in among-region comparisons. The data justify this approach because among-region variation is much greater than within-region variation (*e.g.,* for anterior shield length, an analysis of variance with samples nested within regions yields a region effect, F = 327 df = 5, which is much greater than the effect of samples nested within regions, F = 28 df = 36).

Long Island Sound, the smallest designated region (see Fig. 1), encompasses the boundary between the rocky New England habitats where *L. littorea* abounds and the sandy estuarine habitats farther south where *L. littorea* is very rare (Vermeij, 1978). There is considerable variation in the abundance of *L. littorea* snails and shells between the north and south shores of Long Island Sound. For some analyses, Connecticut and Long Island shore samples are considered separately, though free larval exchange is likely (see Discussion).

Morphological measures

After collection each sample was preserved in 10% formalin, 90% seawater. Each crab's shell species was noted and shell length (at maximum parallel to the columellar axis) and width (at maximum perpendicular to the columellar axis) were measured

TABLE 1

Sites, dates, sizes, and regional groupings of geographic samples[1]

Region	Site	Date	n
Northern Massachusetts	Casco Bay, ME	3 September 1985	8
	Nahant, MA	23 September 1983	112
		29 August 1985	204
	Quincy, MA	4 September 1985	78
Southern Massachusetts	Martha's Vineyard, MA	13 August 1984	133
	Charlestown, RI	14 August 1984	179
Long Island Sound	Guilford, CT	18 September 1983	157
		8 September 1984	160
	Cold Spring, NY	20 September 1983	54
		26 August 1984	92
		14 July 1985	33
		8 October 1985	38
New Jersey	Barnegat Bay, NJ	4 September 1983	79
		20 August 1984	59
		16 September 1985	57
	Corson's Inlet, NJ	16 September 1984	306
Virginia	Chinoteague, VA	19 September 1984	135
	Cherrystone, VA	20 September 1984	97
	Oregon Inlet, NC	22 September 1984	14
Carolinas	Beaufort, NC	25 September 1984	170
	Topsail Inlet, NC	26 September 1984	93
	Southport, NC	27 September 1984	102
	Murrell's Inlet, SC	28 September 1984	115
	Folly Beach, SC	30 September 1984	28

[1] Samples from 1980–1982 are described in Blackstone (1985) and have been deposited in the collections of the Division of Invertebrate Zoology, Yale Peabody Museum, nos. 8120–8137. Samples from 1983–1985 have been deposited in the collections of the Academy of Natural Sciences of Philadelphia, nos. CA4566–CA4584. All sample regions and sites from 1980–1985 are shown in Figure 1; note that not all sites are from the states used to designate the regions.

with a vernier caliper. A categorical index of shell height was calculated by shell length/width (0 = low-spired, ratio less than 1.5; 1 = high-spired, ratio greater than 1.5; this is a somewhat arbitrary designation, but it separates the shells used by *P. longicarpus* in the study areas into recognizably low-spired and high-spired species, see Blackstone, 1985, and spire height is related to shell preference, see below). A categorical index of shell fit was also noted (0 = shell fit adequate to protect crab, crab able to retract fully and form an operculum with its chelae, or 1 = shell fit inadequate because of small size or structural damage, crab unable to fully retract). Clear determination of this index was possible in almost all cases because *P. longicarpus* preserved in 10% formalin die retracted fully into their shells (unless the shell was so small that retraction was impossible). The few individuals intermediate in shell fit were excluded from further analysis. If possible the crab was removed from its shell by gently pulling its appendages; usually removal was not possible and the shell was cracked in a small vice. The crab was sexed by pleopodal morphology. The hard part of the carapace (anterior shield length, Fig. 2) was measured using a dissecting microscope equipped with an ocular micrometer. The total length of the right cheliped (Fig. 2) was then measured with a vernier caliper unless this appendage had been recently regenerated. (A recently regenerated cheliped is much shorter than the pereiopods and has an atypical color.) For some of the earlier samples, right chela length

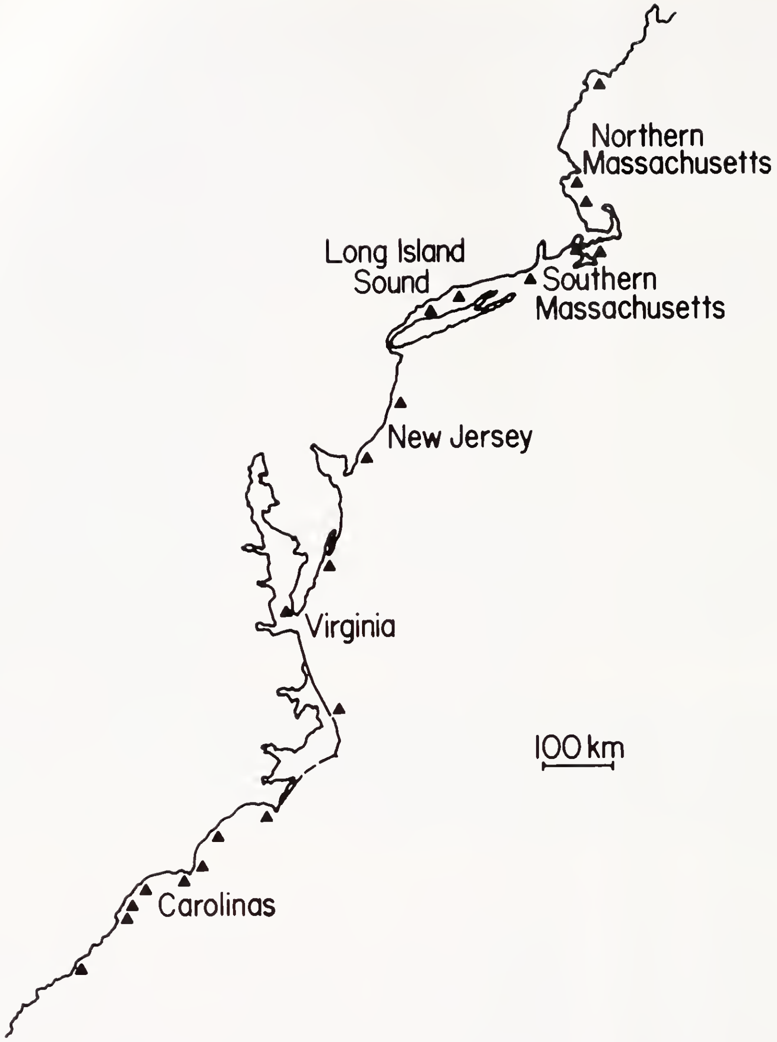

FIGURE 1. The Atlantic coast of North America from Maine (approx. 70° W. lat., 44° N. long.) to Georgia (approx. 80° W. lat., 33° N. long.) showing the regions and sites sampled. Sites (triangles) include those from 1980–1982 (see Blackstone, 1985) and 1983–1985 (Table 1). Rocky substrata and periwinkle snails are common northward from the northern shore of Long Island Sound. Primarily sandy substrata from Long Island southward to Virginia support few periwinkles; south of Virginia periwinkles do not occur.

instead of cheliped length was measured. This measure shows the same pattern as right cheliped length (Blackstone, 1985), and only the cheliped data are presented.

Anterior shield length is a measure of carapace and cephalothorax size in *P. longicarpus*. Since anterior shield lengths show slightly non-normal distributions and correlations between sample means and variances, a natural logarithmic transformation was done to better meet the assumptions of analysis of variance. Cheliped allometry was measured by double logarithmic, ieast-squared regressions of right cheliped

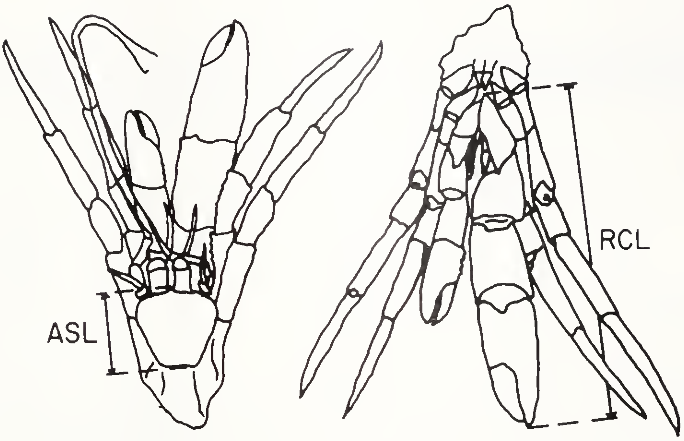

FIGURE 2. Camera lucida tracings of the calcified parts of a *Pagurus longicarpus* hermit crab. Anterior shield length (ASL) is shown on the dorsal tracing, while right cheliped length (RCL) is shown on the ventral tracing.

length on anterior shield length. The high correlation coefficients obtained indicate that other bivariate linear methods would yield similar results (Yates, 1950; Gould, 1966; Rayner, 1985). The slope of these regressions represents the relationship between the specific growth rates of the two parts (Huxley, 1932; Teissier, 1960; Laird, 1965; Shea, 1985). If this slope is greater than unity, the right cheliped has a faster specific growth rate than the anterior shield. Differences among regions were tested using analysis of covariance tests for homogeneity of slopes.

Estimating shell availability

Samples of empty shells not only are rarely obtainable, but cannot be used to measure shell availability with certainty (Wilber and Herrnkind, 1982; Blackstone and Joslyn, 1984). Abundances of living gastropods have different implications depending on the ecological circumstances (Spight, 1977; Wilber and Herrnkind, 1982). Here, data on shell use provide insight into shell availability as perceived by *P. longicarpus.* Observed shell use reflects perceived shell availability, shell preference, and ecological factors (Reese, 1962, 1969; Young, 1979). Studies of the shell preferences of *P. longicarpus* (Mitchell, 1975; Blackstone, 1984; Blackstone and Joslyn, 1984) indicate: (1) preferences for shells that provide adequate fit (defined above), and (2) preferences for high-spired shells (defined above) at small sizes and low-spired shells at larger sizes (greater than 3.3 mm anterior shield length). Patterns of shell use from geographic samples may thus be interpreted; if a crab occupied a shell which is not of the preferred fit or shape, that crab likely perceived a deficiency in the available shell resource. Ecological factors will affect estimates based on shell fit, since hermit crabs in ill-fitting shells are more susceptible to predators and other agents of mortality (Reese, 1969; Vance, 1972b). Estimates based on shell shape should be less affected by ecological factors. Because hermit crabs switch shells frequently, these measures of perceived shell availability must be made at a populational level. Also, this method implies that shell preference is relatively constant in geographic populations of *P. longicarpus;* while available data largely support this interpretation (Blackstone, 1984), this question could be investigated further.

TABLE II

Regional differences in anterior shield length, cheliped allometry, and use of Littorina littorea *shells in male* Pagurus longicarpus

Region	Shield length Mean	SD	(n)	Cheliped allometry[1] Slope	SE	r^2	(n)	*L. littorea* Inhabited
Carolinas	2.50	0.61	(584)	1.32	0.02	0.96	(269)	0%
Virginia	3.14	0.54	(113)	1.34	0.03	0.95	(98)	0%
New Jersey	4.22	0.82	(420)	1.29	0.02	0.93	(258)	5%
Long Island Sound	2.96	0.72	(1087)	1.26	0.02	0.93	(448)	27%
Southern Massachusetts	3.77	0.94	(219)	1.21	0.02	0.96	(139)	79%
Northern Massachusetts	4.88	1.39	(509)	1.21	0.01	0.97	(346)	62%

[1] Linear regression of the natural logarithm of the right cheliped length on the natural logarithm of the anterior shield length. Regression slope, standard error of slope, correlation coefficient, and sample size are shown. (Sample sizes for cheliped allometry are smaller than those for shield length; many crabs from all samples had missing or regenerate chelipeds; also some early samples were measured for right chela, rather than cheliped, length.)

RESULTS

Morphological variation

There is significant among-region variation in anterior shield length in male *P. longicarpus* (ANOVA, F = 327, df = 5, $P < .0001$), but this variation does not correlate with the use of *L. littorea* shells (Table II). For instance, in New Jersey, *P. longicarpus* are large, but inhabit few *L. littorea* shells. On the other hand, the cheliped allometry of male *P. longicarpus* shows an inverse correlation with the use of *L. littorea* shells (Table II). In regions where *L. littorea* is rare (Carolina, Virginia, New Jersey), the right cheliped grows significantly faster relative to the anterior shield than in regions where *L. littorea* is commonly used (Massachusetts) (ANCOVA, $P < .01$).

In Long Island Sound there are within-region differences in the abundance of periwinkle snails and their shells. On the northeast (Connecticut) shore, periwinkles are abundant on the primarily rocky substrata, while on the southwest (Long Island) shore, periwinkles are scarce on the primarily sandy substrata (Vermeij, 1978). A comparison of sites from these two shores (Fig. 3) also shows an inverse correlation

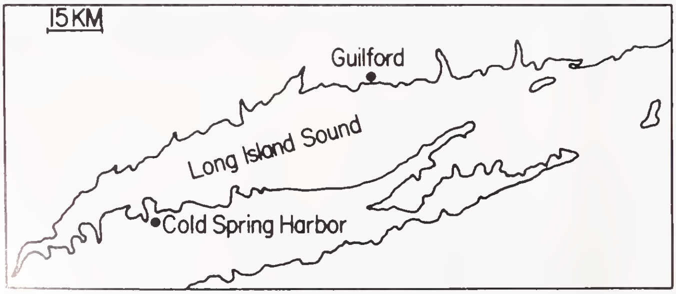

FIGURE 3. Long Island Sound showing the two sites sampled. The northeast Connecticut shore (Guilford) is primarily rocky substrata and periwinkle snails are abundant, while the southwest Long Island shore (Cold Spring Harbor) is primarily sandy substrata and periwinkles are rare.

TABLE III

Micro-geographic and historical differences in cheliped allometry and use of Littorina littorea *shells for male* Pagurus longicarpus *from Long Island Sound*

	Cheliped allometry[1]				
	Slope	SE	r^2	(n)	*L. littorea* Inhabited
Guilford, Connecticut	1.21	0.02	0.92	(358)	32%
Cold Spring, Long Island	1.38	0.06	0.86	(90)	5%
Long Island Sound (1873)	1.32	0.05	0.91	(72)	0%

[1] As in Table II.

between relative growth and the use of periwinkle shells (Table III). Guilford, Connecticut, crabs differ significantly from Cold Spring Harbor, Long Island, crabs both in relative cheliped growth ($P = 0.009$, ANCOVA test for homogeneity of slopes) and in use of *L. littorea* shells (*L. littorea:* native shells, 286: 618, 10: 173, Guilford and Cold Spring respectively; G-test of independence = 66.7, df = 1, $P < 0.0001$).

The observed relationship between reduced relative growth and use of *L. littorea* shells prompted a search for pre-*L. littorea* historical samples of *P. longicarpus* (see Appendix). The only such sample large enough to be useful was from Long Island Sound (exact location unknown). The relative cheliped growth of the pre-*L. littorea* males from Long Island Sound is not significantly different from that of modern males from Long Island, New Jersey, and more southern regions (ANCOVA, $P > .25$). However, these pre-*L. littorea* males show faster relative cheliped growth than Connecticut or Massachusetts males (Table III; ANCOVA, $P < .05$).

Estimates of shell availability

The low-spired shells of adult periwinkles are intermediate in size between those of the small native snails (*Ilyanassa, Nassarius, Urosalpinx*) and large native snails (*Polinices*) (Fig. 1 in Blackstone, 1985). Further, periwinkles may have reduced the abundance of the small, high-spired native mud snails (*Ilyanassa* and *Nassarius*) either by direct competition (Brenchley and Carlton, 1983) or by habitat alterations (Bertness, 1984). In areas where periwinkles are abundant, it is expected *a priori* that (1) medium-size, low-spired shells are more abundant, and (2) small, high-spired shells are less abundant.

The results of estimates of shell availability largely confirm these expectations (Fig. 4). Small male *P. longicarpus* (less than 3.3 mm anterior shield length) always inhabit shells which fit, but in regions where periwinkle shells are abundant (Connecticut and Massachusetts), they often inhabit low-spired shells, the less preferred shape at these sizes (Mitchell, 1975; Blackstone, 1984; Blackstone and Joslyn, 1984). This suggests that the availability of small, high-spired shells is lower in New England than in southern regions.

Shell availability estimates for medium-size male *P. longicarpus* (anterior shield length 3.3–4.9 mm) also show geographic variation. In the Carolinas and Virginia these crabs all inhabit shells which fit, but more than half inhabit high-spired shells, the less preferred shape at these sizes (Mitchell, 1975). In these regions, the low-spired shells of the large native littorine, *Littorina irrorata,* are an important component of the preferred shell resource, while *L. littorea* shells do not occur or are very rare.

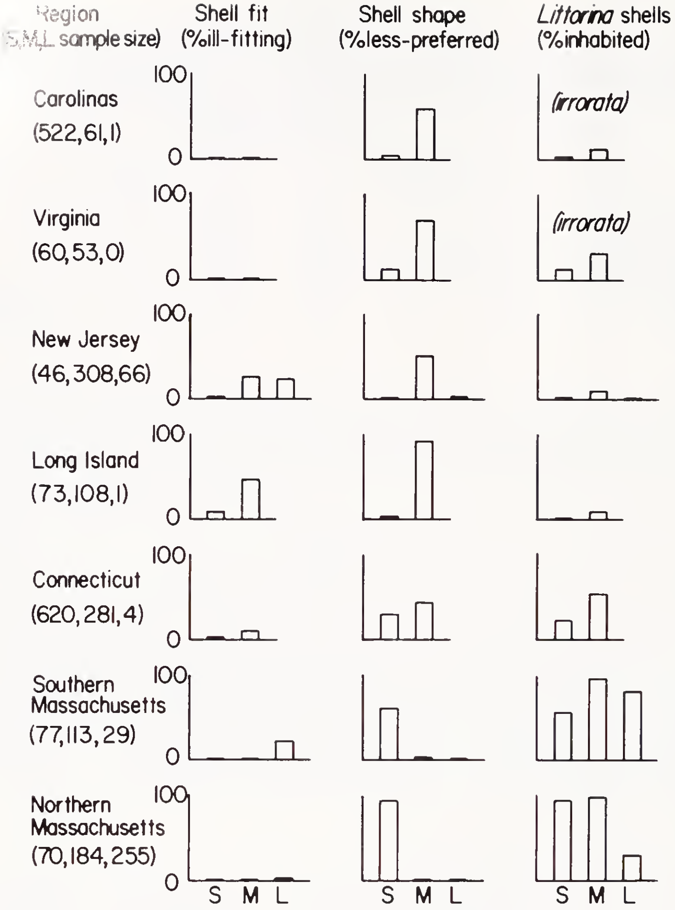

FIGURE 4. For six geographic regions (Long Island Sound is divided into Long Island and Connecticut, see Fig. 3), indices of shell fit and shell shape provide estimates of shell availability and the impact of *Littorina littorea* shells on small (S; less than 3.3 mm anterior shield length), medium (M; between 3.3 and 4.9 mm ASL), and large (L; greater than 4.9 mm ASL) *Pagurus longicarpus* males. In the Carolinas and Virginia, shells of *Littorina irrorata* (plotted), a native snail, are an important part of the shell resource, but many medium-size crabs inhabit shells of the less preferred shape. In New Jersey and Long Island, *L. irrorata* is very rare or does not occur and *L. littorea* (plotted) is also rare; many medium-size crabs inhabit shells which do not fit and many inhabit shells of the less-preferred shape. In New England (Connecticut and Massachusetts), hard substrata and *L. littorea* (plotted) are common and: (1) more medium-size crabs inhabit shells which fit than in New Jersey and Long Island (G-tests, $P < .001$), (2) fewer medium-size crabs inhabit shells of the less-preferred shape than in Long Island and more southern regions (G-tests, $P < .001$), and (3) more small crabs inhabit shells of the less-preferred shape than in Long Island and more southern regions (G-tests, $P < .001$). (Samples sizes <5 are not plotted.)

In New Jersey and Long Island, many medium-size specimens of *P. longicarpus* occupy shells of less than preferred fit or shape. *Littorina irrorata* shells are very rare or do not occur in these areas (Vermeij, 1978), while *L. littorea* shells are inhabited by 8% of the medium-sized crabs (Fig. 4). On the Connecticut shore of Long Island Sound, significantly more *L. littorea* shells are occupied by medium-size crabs (52%, G-test, $P < .0001$), and both indices of shell fit and shell shape indicate that preferred shells are more available to medium-size crabs in this area than in southern Long Island Sound and New Jersey.

In Massachusetts, virtually all medium-size specimens of *P. longicarpus* occupy *L. littorea* shells and very few crabs occupy less than preferred shells. Even in northern Massachusetts where large, low-spired shells of native *Polinices* species are occupied by many large males (>4.9 mm anterior shield length; Fig. 4), medium-size crabs occupy *L. littorea* shells almost exclusively (97%, Fig. 4).

DISCUSSION

The introduction of *L. littorea* and the subsequent availability of its medium-size, low-spired shells may have significantly improved shell availability as perceived by medium-size male *P. longicarpus* specimens in New England. A discontinuity in the native shell resource is apparent between small and large species of shells (Blackstone, 1985). Medium-size crabs are most likely to perceive this discontinuity. Indices of perceived shell availability calculated for these crabs suggest that the introduced periwinkle shells improve shell availability for this size class. While one index (shell fit) could easily be influenced by ecological factors, the other (shell shape) is much less sensitive. Both indices suggest that medium-size male *P. longicarpus* perceive shell availability as high only in areas where *L. littorea* shells are very common. Again, geographic variation in shell preference could affect this conclusion, but available data (Mitchell, 1975; Blackstone, 1984) suggest that shell preference is rather constant in *P. longicarpus* along the Atlantic coast.

Reduced shell availability, or more precisely, an increased incidence of ill-fitting shells in a hermit crab population, is associated with increased cheliped allometry. Laboratory experiments suggest that *P. longicarpus* specimens which inhabit shells of an inadequate fit tend to exhibit faster relative growth of the right cheliped than those which inhabit shells of adequate fit (Blackstone, 1985, 1986). The observed geographic pattern of cheliped allometry thus may be related to the data on shell fit.

In the Carolinas and Virginia, *P. longicarpus* males exhibit a high rate of relative cheliped growth but do not inhabit shells of inadequate fit. While the shell shape index suggests that medium-sized crabs perceive deficiencies in the shell resource, ecological factors might prevent crabs in ill-fitting shells from remaining in the population. Populational samples do not contain crabs which experienced ill-fitting shells, and such samples will not show environmentally induced acceleration of relative growth. The high rate of cheliped allometry in these regions probably stems from genetic factors, and laboratory experiments support this interpretation (Blackstone, 1985, in press).

In New Jersey and Long Island, *P. longicarpus* males exhibit a high rate of relative cheliped growth and also inhabit shells of inadequate fit. Populational samples include many crabs which experienced ill-fitting shells (measures of shell fit are best interpreted at the population level, since these crabs switch shells frequently). The high rate of cheliped allometry in these areas may stem from environmentally induced acceleration of relative growth.

In Connecticut and Massachusetts, *P. longicarpus* males exhibit a low rate of relative cheliped growth and seldom inhabit shells of inadequate fit. Data from Long Island Sound suggest that this low rate of cheliped allometry may stem from the introduction and use of periwinkle shells.

In Long Island Sound, *P. longicarpus* are probably homogeneous for genetic factors controlling cheliped growth, because hydrographic patterns indicate that planktonic larvae should be well dispersed (see Riley *et al.*, 1959). While strong selection could override this gene flow (*e.g.*, Koehn *et al.*, 1976), there is no evidence that strong selection for slight differences in cheliped proportion is operating. Nevertheless, *P. longicarpus* males from the Connecticut shore show reduced relative cheliped growth when compared to those from the Long Island shore and this difference is associated with the use of *L. littorea* shells (Table III). Further, the relative cheliped growth of males from a pre-*L. littorea* Long Island Sound sample is greater than that of modern males from Connecticut, but equal to that of males from Long Island.

Of the possible explanations for these geographic and historical patterns, the most parsimonious one, given the available data, is that the introduction of *L. littorea* increased the shell availability as perceived by male *P. longicarpus* in some northern areas where it became common (Connecticut and Massachusetts), but not in other northern areas where it is still scarce (Long Island and New Jersey). In areas where shell availability was increased, fewer crabs inhabited ill-fitting shells and cheliped allometry decreased. These results may indicate that historical change in allometry can be ecophenotypic. This supports suggestions made by other workers (Fryer *et al.*, 1983, 1985; Kat and Davis, 1983; Kemp and Bertness, 1984; Palmer, 1985).

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APPENDIX

Curators of the following museums provided information on historical collections of *P. longicarpus:* U. S. National Museum of Natural History (Washington), Academy of Natural Sciences (Philadelphia), American Museum of Natural History (New York), Yale Peabody Museum (New Haven), Museum of Comparative Zoology (Cambridge), and British Museum, Natural History (London).

Pre-*L. littorea* specimens of *P. longicarpus* were located for Massachusetts (n = 144) and Long Island (n = 321). Most of the specimens were retracted inside their occupied shells and could not be measured. Thus populational measures (*e.g.*, mean anterior shield length) were impossible. The maximum anterior shield length of specimens out of shells was similar to that found in modern samples (historical: modern; for Long Island Sound, 5.3:6.1 mm; for Massachusetts, 7.7:8.3 mm). This suggests that *P. longicarpus* did not increase in size, particularly when differences in sample sizes are considered (historical:modern; sample sizes for Long Island Sound, 321:1807; for Massachusetts, 144:1060). The shells inhabited by these pre-*L. littorea* hermit crabs are the same as the native species new inhabited.

Measures of cheliped allometry could be taken. These are presented for 72 male crabs from an 1873 Long Island Sound sample (YPM #7935; *L. littorea* arrived in Long Island Sound after 1875; Carlton *et al.*, 1982; Vermeij, 1982; Brenchley and Carlton, 1983). This is the only historical sample with many (>15) specimens out of shells.

Reference: *Biol. Bull.* **171:** 391–404. (October, 1986)

THE ECOLOGICAL ROLE OF DEFENSIVE SECRETIONS IN THE INTERTIDAL PULMONATE *ONCHIDELLA BOREALIS*

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ABSTRACT

Repugnatorial glands located in the marginal papillae of the intertidal ochidiid pulmonate *Onchidella borealis* secrete a viscous fluid in response to mechanical or chemical stimulation. In laboratory encounters, this fluid repels intertidal predatory asteroids, particularly *Leptasterias hexactis,* but not predatory gastropods, polyclad turbellarians, nemerteans, or fishes. Intertidal crabs consume dead *O. borealis* readily, but seldom consume living individuals capable of firing their glands. The vertical range of *O. borealis* overlaps that of *L. hexactis,* whereas limpets that are vulnerable to predation by the sea stars generally live higher on the shore. On a small scale, *O. borealis* and *L. hexactis* occupy similar microhabitats (*e.g.,* crevices, algal holdfasts), but are seldom found together. Field and laboratory experiments suggest that this negative spatial correlation may result from expulsion of the sea stars by onchidiids.

INTRODUCTION

Many marine gastropods lacking protective shells have evolved alternative defenses such as incorporation of cnidarian nematocysts, distasteful chemicals, and aposematic or cryptic coloration. Shelled gastropods often exhibit flight behaviors and other evasive tactics (Bullock, 1953; Feder, 1963; Margolin, 1964; Mauzey *et al.,* 1968; Phillips, 1976). The ecological consequences of molluscan chemical defenses have been tested in relatively few cases (*c.f.* Rice, 1985). In many cases, organisms containing protective chemicals are probably not consumed; the ecological significance of the defense is complete protection from most predators. In other cases, defenses may be effective against some predators and not others.

Stylomatophoran pulmonates in the family Onchidiidae occupy the middle and high intertidal zones of rocky shores throughout many areas of the world. The species that have been studied breathe air and are most active at low tide on overcast days (Arey and Crozier, 1921; Pepe and Pepe, 1985). Many species return to home sites during high tide (Pepe and Pepe, 1985; McFarlane, 1980). The margins of the snails are endowed with a row of multicellular glands that secrete a white, viscous fluid. These glands were named repugnatorial glands in *Onchidium floridana* because they were thought to repel fishes and crabs (Arey, 1937; Arey and Crozier, 1921). A unique organic molecule isolated from the glands of *Onchidella binneyi* has been described

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recently (Ireland and Faulkner, 1978), and is thought to be responsible for the observed repulsion.

Studies dealing with the effectiveness of molluscan defenses have mostly dealt with escape responses of shelled gastropods (Clark, 1958; Feder, 1963; Engstrom, 1982; McKillup, 1982; Schmidtt, 1982; Garrity and Levings, 1983). The chemical defenses of molluscs have been less studied (Thompson, 1960; Edmunds, 1966; Ambrose *et al.,* 1979; Rice, 1985). Compounds isolated from molluscs have been tested for a repugnatorial effect in a few cases (Ireland and Faulkner, 1978; Thompson *et al.,* 1982). However, the effects of molluscan defenses on the ecology of potential predators has never been investigated.

In this study we document the distribution of *Onchidella borealis* in the intertidal zones of Washington and British Columbia, describe the outcomes of encounters between *O. borealis* and various potential predators, and present preliminary evidence that the defensive secretions can modify the distributions of predatory asteroids. Aside from earlier anecdotal work, there have been no studies of predator-prey interactions involving onchidiids, and to our knowledge, repellant defensive secretions do not influence predator distributions in any other animal species.

MATERIALS AND METHODS

Onchidella borealis were studied on San Juan Island, Washington, and in Barkley Sound, Vancouver Island, British Columbia, from 1973 to 1985. Distributions were documented quantitatively at several sites on the west side of San Juan Island (Marvista Resort, Cattle Point, Eagle Cove, Pile Point) and on Seppings Island in Barkley Sound. Animals for laboratory and field experiments were collected from all of these sites and also from a high density population occupying a cobble field in the intertidal zone of Grappler Inlet, British Columbia.

Onchidella borealis were maintained in the laboratory in glass bowls, beakers, or plastic refrigerator containers covered loosely with plastic lids or cheesecloth. Snails quickly crawled out of containers without covers. Most snails died if they were submerged for more than a day; we obtained highest survival by maintaining snails with damp algae (*Ulva* sp.) or moist paper towels, not submerged in seawater.

Distribution of Onchidella borealis *and associates*

Vertical zonation was documented at several sites with transects of 50 by 50 cm quadrats. The profile of the shore was measured with a meter stick and line level. At one site, Eagle Cove, the large quadrats were subdivided into 10 cm plots to investigate the small-scale spatial distributions of *O. borealis* and one of its potential predators, *Leptasterias hexactis.*

Encounters between Onchidella borealis *and potential predators*

We documented responses of potential predators to *O. borealis* by observing individual encounters staged in glass bowls of seawater. Predators used included polyclad flatworms (*Notoplana atomata*), nemerteans (*Paranemertes peregrina*), gastropods (*Searlesia dira, Thais lamellosa*), anomuran (*Petrolisthes cinctipes*) and brachyuran (*Hemigrapsus nudus*) crabs, fishes (*Gobiesox meandricus, Apodichthys flavidus*), and asteroids (*Leptasterias hexactis, Henricia leviuscula, Pisaster ochraceous, Solaster stimpsoni*). All predators were collected from intertidal areas where *O. borealis* occurred. In instances where the predators showed distinct avoidance of *O. borealis,* the experiments were repeated with freshly killed (by drowning) *O. borealis* to determine whether the predators responded to the presence of the snail body *per se,* or if repugnatorial gland secretions produced by living snails were necessary to initiate the response.

After observing several encounters between an *O. borealis* and a potential predator, we determined the best way to qualitatively score the encounter. For the worms and the snails, we scored any obvious changes in direction of movement after touching *O. borealis* as a response. The data were thus of a binary (response/no response) nature. Asteroids demonstrated both directional and postural changes, so their responses were divided into four easily recognized categories that could be ranked according to the degree of response. Responses of all potential predators to *O. borealis* were cast in 2-factor or 3-factor contingency tables and analyzed by log-likelihood statistics (Sokal and Rohlf, 1981).

In some experiments, we noted responses of predators to small amounts of repugnatorial gland secretion rather than to the predator itself. Repugnatorial gland secretion was obtained by placing a Pasteur pipette over a repugnatorial gland and probing the animal gently with the pipette until the gland discharged. The secretion was expelled by the snail into the pipette where it remained until use. Because the secretion is more viscous than seawater, it was easily visible when discharged from the pipette. Once used, individual specimens of *O. borealis* were returned to a common container and drawn haphazardly for each set of experiments; thus, individual specimens of *O. borealis* were used in more than one trial. At least 15 minutes elapsed between trials. A given set of animals was never used for more than 2–3 consecutive days before being returned to the field.

Individual behavioral interactions between *O. borealis* and crabs or fishes were difficult to document because of the rapid movements of the predators and the large size disparity between predator and prey. Where these large motile predators were involved, we exposed *O. borealis* to predators in cheesecloth-covered bowls for 5 hours, then noted the number of prey consumed or damaged. Three *O. borealis* and one predator were used in each bowl. Predators were acclimated to laboratory conditions for 3–3.5 hours before the experiments.

Experiments on the Leptasterias/Onchidella *interaction*

Additional work was undertaken with the asteroid predator *Leptasterias hexactis,* because it regularly occurs in the same tidal zone as *O. borealis* and shows a dramatic response to repugnatorial gland secretions. Laboratory and field feeding experiments were done to determine which common intertidal gastropods were consumed by the starfishes. In the laboratory, 10 each of *O. borealis* and the limpets *Collisella digitalis* and *Notoacmaea scutum* were placed in a bowl with a single starfish. We noted the prey species eaten and replaced individuals as they were consumed, thus the relative proportions of potential prey were constant throughout the experiment. The experiment was repeated twice. A similar experiment was run in the field. The experimental containers used in the field consisted of plastic jars glued to bricks and placed in the mid intertidal zone. Each jar had a cover of nitex screen and was positioned with the lid down so the jar would drain at low tide. Because of the small sizes of the jars (500 ml), only two of each prey item were placed in each jar. The experiments were monitored daily during the spring tidal sequence, but were not monitored during neap tides, when they were exposed for only short periods. Only 2 of 12 jars survived the entire experiment; the rest were damaged or removed by the surf.

Onchidella borealis and *L. hexactis* commonly occupy holdfasts of the brown alga *Hedophyllum sessile* in the mid-intertidal zone. We investigated the possibility that the repugnatorial glands of *O. borealis* can repel starfish and thereby modify the distributional patterns of the latter. Starfish and snails were seeded into previously defaunated holdfasts (in the lab and field), then emigrations were noted. *Hedophyllum sessile* plants containing neither *L. hexactis* nor *O. borealis* were collected from Pile Pt., San Juan Island. The blades of the plants were removed and the holdfasts were rinsed in fresh water for at least 12 hours to remove all macroscopic organisms. Holdfasts were then transferred to running seawater aquaria, where they showed no signs of decomposition for several days. Holdfasts were divided into two separate aquarium compartments. Small *L. hexactis* were added to each compartment and allowed to enter the holdfasts. After the starfish had colonized both holdfasts, the holdfasts were carefully removed from the water and 20 to 25 *O. borealis* were added to one of them, the other serving as a control. The holdfasts were then placed in separate compartments of empty aquaria and seawater was trickled around them to keep them moist. After two hours, the number of *L. hexactis* that had left each holdfast was recorded. The experiment was repeated six times.

The holdfast experiment was repeated under field conditions. Individual *L. hexactis* were added (during low tide) to small clumps of *H. sessile* holdfasts located between 0.0 m and +1.0 m above MLLW. Five clumps without *O. borealis* served as controls and seven clumps containing *O. borealis* served as treatments. The holdfasts were censused after 24 hours (two tidal sequences) and the number of *L. hexactis* present at each holdfast was recorded. The holdfasts were then removed and examined once again for *O. borealis.*

RESULTS

General observations

In *Onchidella borealis,* the number of repugnatorial glands varies as a function of animal size; large individuals may have more than 20 glands (Fig. 1). Internally, each gland consists of many flask-shaped or columnar secretory cells surrounding a lumen, covered by a thick muscle sheath, and embedded in connective tissue. The lumen of each gland opens to the outside on a distinct, individual marginal papilla (Fig. 1). The ultrastructure of the glands and surrounding structures will be described in a second paper (Greenwood and Young, in prep.). When stimulated mechanically (*e.g.,* with a glass probe) on the dorsum, *O. borealis* may direct its papillae toward the stimulus, but it does not fire until the tip of a papilla is stimulated directly. Upon direct stimulation, the contacted gland releases a milky transluscent, viscous substance that is clearly visible; the repugnatorial secretion may extend several millimeters from the animal. Each gland seems to function as an independent effector. When stimulated lightly, a gland does not always discharge the entire contents of its lumen, but can fire repeatedly. However, in other cases, particularly when responding to stimulation by starfish, each gland fires only once, then requires a "recycling period" before it is capable of firing again. The glands appear to require less mechanical stimulation to elicit their firing when probes are coated with starfish mucus, but this was not quantified.

Distribution of Onchidella borealis *and associates*

Populations of *O. borealis* were found on the west side of San Juan Island where rocky shores are exposed to the moderate wind-generated surf of the Straits of Juan de Fuca and the Rosario Straits. Individuals were only rarely found on the east side of the island where tidal currents are strong and waves are generally

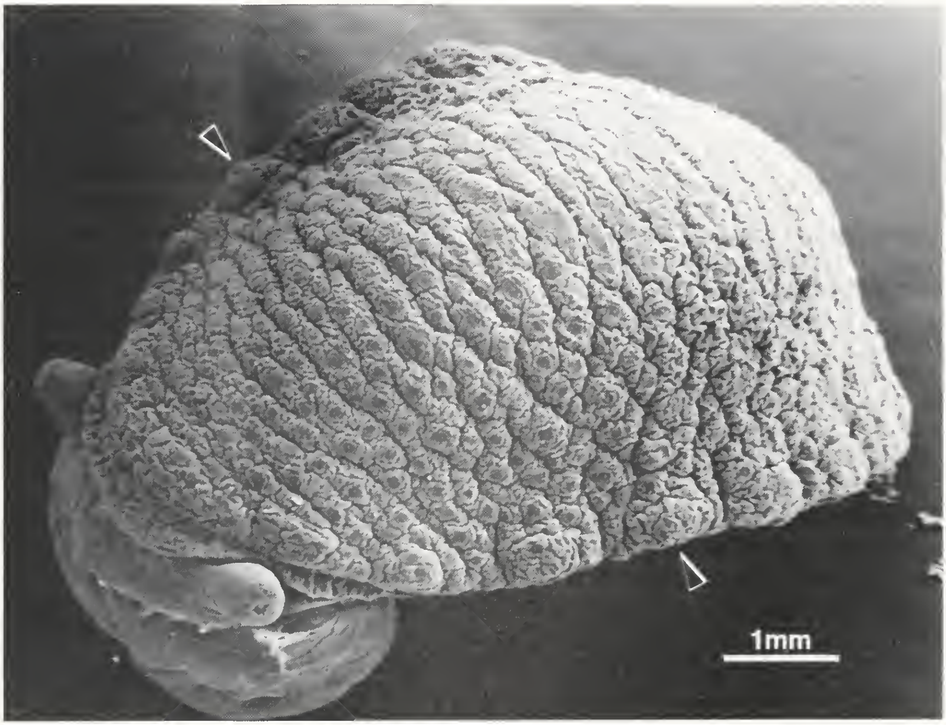

FIGURE 1. Scanning electron micrograph of *Onchidella borealis*, showing marginal papillae (arrow) where repugnatorial glands are located.

small. In Barkley Sound, populations were found on most coasts with moderate exposure. Surprisingly, the highest population density we have encountered is in a very protected cove of Grappler Inlet. There, *O. borealis* occurred abundantly on rocks in the middle intertidal zone, but also on *Ulva* sp. and cobbles overlying mud in the lower intertidal. Comparable habitats in the San Juan Islands never supported populations of *O. borealis.*

On rocky shores at low tide, *O. borealis* primarily occupy crevices, empty barnacle shells, and holdfasts of *Hedophyllum sessile* and *Laminaria* spp. On rainy or foggy days, many individuals leave their shelters and wander over the rock. Figure 2 shows the vertical distribution of *O. borealis,* several co-occuring limpets, and the predatory starfish *Leptasterias hexactis.* The precise tide level occupied by *O. borealis* varies from site to site, but is always between about −0.5 m and +1.5 m above MLLW. This zone overlaps the distribution of *L. hexactis,* though the two populations do not always peak at the same level. At most sites, large starfish were found lower on the shore than *O. borealis,* and the asteroids primarily occupied the larger crevices. Small starfish also ocur in crevices, but live in relatively large numbers in holdfasts of *H. sessile* and various laminarians as well.

At Eagle Point, all individuals of both species were counted within 10 cm quadrats. There was a non-significant, though negative correlation between the occurrence of the two species. In general, no *L. hexactis* were found in quadrats containing high densities of *O. borealis.* The same held true for *H. sessile* holdfasts. At Pile Point, San Juan Island, two sites, one more exposed than the other, were examined in detail. At

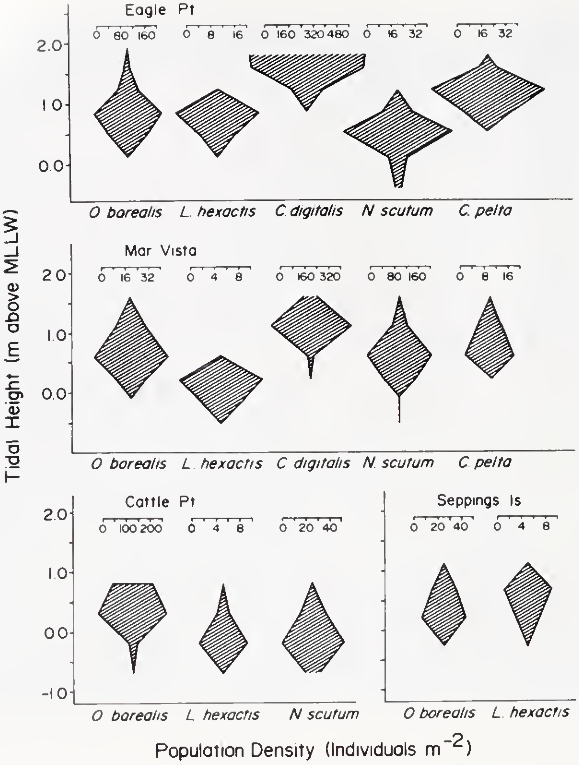

FIGURE 2. Vertical distributions of *Onchidella borealis, Leptasterias hexactis,* and associated limpets at four intertidal sites. Seppings Island is in Barkley Sound, British Columbia. All other sites are on the west side of San Juan Island, Washington state.

the more exposed site, only one *L. hexactis* was found, next to a holdfast. An average of 28.6 *O. borealis* (range: 13–64; n = 8) occurred in each holdfast. At the less exposed site, which was 200 m from the other site, higher densities of *L. hexactis* were found. *Leptasterias hexactis* in holdfasts were significantly smaller (mean = 12.9 mm; n = 9) than individuals occurring in cracks (mean = 44.3 mm; n = 12; $P < 0.001$, Mann-Whitney U-Statistic: Sokal and Rohlf, 1981). At the less exposed site, *O. borealis* were found primarily in shallow cracks above the level of the *L. hexactis.* Only one holdfast contained any *O. borealis.* The two species were never found within the same holdfast at either site.

Limpets, which occurred abundantly at all sites, showed a characteristic zonation pattern (Fig. 2). *Notoacmaea scutum* occupies virtually the identical zone as *O. borealis.* The lower limit of *Collisella pelta* is slightly higher than that of *O. borealis* and *N. scutum,* though the upper limit is at approximately the same level. *Collisella digitalis* overlaps the other species slightly, but peaks in its distribution much higher than *O. borealis, L. hexactis,* or any of the other limpets. These distributions suggest that all of the mollusc species except possibly *C. digitalis* should be available to *L. hexactis* as food.

Predatory invertebrates occurring in the same zones as *O. borealis* included asteroids, decapod crustaceans, fish, small polychaetes, nemerteans, and thaiid gastropods. Predation on *O. borealis* was never observed in the field.

TABLE I

Response of the polyclad flatworm Notoplana atomata *to a dilute solution of repugnatorial gland secretion applied dorsally and ventrally*

Substance applied	Surface	Response	No response
R. G. secretion	Dorsal	17	3
	Ventral	5	15
Seawater	Dorsal	0	20
	Ventral	0	20

A response consisted of "writhing" or active undulation of the margin. All of the two-way interactions are significantly different from random (Three-way log-likelihood [G] test: Sokal and Rohlf, 1981).

Encounters between Onchidella borealis *and potential predators*

Specimens of *Onchidella borealis* showed no response to *Notoplana atomata* and were never seen to fire their glands in response to the flatworm. Thick mucus removed from the ventral surface of the worms with a probe also did not elicit firing, though the snails curled their bodies in response to the mucus application. *Notoplana atomata* specimens demonstrated no apparent behavioral changes when encountering *O. borealis* in the laboratory (n = 50 trials with 5 individuals). Likewise, they seldom responded when placed in a small pool of seawater containing repugnatorial gland secretion (hereafter referred to as RGS). When RGS was applied dropwise to the dorsal surface, however, the worms undulated their margins rapidly (Table I). All of the two-way interactions in the contingency table were significant (Table I). Thus, RGS produces a different response than seawater, and worms respond differently to application of solution to the dorsal surface than to ventral application.

The hoplonemertean *Paranemertes peregrina,* which commonly occurs with *O. borealis* in Grappler Inlet, generally did not change course when encountering *O. borealis* in its path (4 course changes in 50 trials). The worms hesitated slightly when first encountering the snails, then resumed their course. The hesitation observed was not different from that seen when a worm encountered an inanimate object such as the blunt end of a pin. As *P. peregrina* dragged its body across the margin of *O. borealis,* the latter fired repugnatorial glands, but the secretions had no apparent effect on the speed or direction of locomotion of the worm. Worms were induced to respond (by changing their paths) by pipetting concentrated RGS directly on the head (27 course changes in 35 trials); seawater pipetted in an identical manner as a control had no effect (0 course changes in 35 trials). The interaction between the stimulus (RGS *vs.* seawater) and response (course change *vs.* no course change) was significant ($G = 66.986$, 2 d.f., $P < 0.001$).

Individuals of the gastropod *Searlesia dira* (4.56 ± 2.16 g wet wt.; variances reported here and subsequently are 1 Standard Deviation) altered their crawling paths in only 3 of 25 encounters with *O. borealis. Thais lamellosa* (13.82 ± 1.11 g wet wt.) changed its path in 4 of 25 staged encounters. More often, the shelled snails would crawl over *O. borealis,* or *O. borealis* would crawl up on the shell of the snail. Repugnatorial gland secretion applied to exposed regions of the head of the two gastropod species elicited retraction in 3 of 25 trials. Seawater elicited a similar retraction once in *S. dira.* The repugnatorial glands did not fire in most encounters between shelled gastropods and *O. borealis.*

The anomuran crab *Petrolisthes cinctipes* (range of wet weights: 0.72 g to 3.56 g) never consumed *O. borealis* in our experiments. Pooled results of the 5-hour experi-

TABLE II

Live and dead Onchidella borealis *consumed, damaged, and not eaten by* Hemigrapsus nudus

Prey condition (beginning)	Prey condition		
	Intact	Eaten	Damaged
Live	43	8	9
Dead	28	21	11

Data are pooled from 20 replicate pairs of dishes, each dish containing either three live or three dead *O. borealis* and one crab at the outset. The distribution of counts within the table differs significantly from random ($G = 9.44384$; $P < 0.01$).

ments with *Hemigrapsus nudus* (2.78 ± 1.00 g wet weight) are given in Table II. A few live *O. borealis* were eaten or damaged by the crabs, but significantly more dead ones were consumed. This suggests that although the defensive secretions of *O. borealis* are not completely effective against *H. nudus,* they probably reduce mortality. In many of the cases where animals were partly consumed (damaged), the foot and soft internal viscera were consumed and the mantle margin, where repugnatorial glands are located, was left intact.

The intertidal fishes used in laboratory trials, *Gobiesox meandricus* and *Apodichthys flavidus,* measured 7.3 ± 2.8 cm and 11.2 ± 3.6 cm long, respectively. In two identical runs with each species, each lasting five hours, and including five *Onchidella borealis,* none was eaten or mutilated.

The most dramatic responses to *O. borealis* were demonstrated by small asteroids. Total armspans of the individuals used are as follows: *Leptasterias hexactis,* 4.68 ± 0.54 cm; *Pisaster ochraceous,* 5.75 ± 1.77 cm; *Henricia leviuscula,* 9.85 ± 1.91 cm. Figure 3 shows a typical encounter sequence between *L. hexactis* and *O. borealis.* The glands of *O. borealis* almost always fired upon first contact with an asteroid tube foot. Often the margins of the mantle were flexed so that the gland openings were pointed upward toward the tube foot. Only the glands in the immediate vicinity of the tube foot fired. When the secretion touched the asteroid, the starfish began moving in another direction while simultaneously lifting the affected ray. Within the next few seconds, the ray was lifted to a vertical position, or sometimes flexed completely so that the aboral surface of the ray contacted the aboral surface of the disk. The responses of three asteroid species to live and dead *O. borealis* are given in Table III. In general, the starfish did not respond to dead snails and there were interspecific differences in the level of response to live snails (Table IV). *Henricia leviuscula* responded in the same manner as *L. hexactis* and *P. ochraceous* but less frequently and less dramatically. On one occasion, we exposed a small (20 cm diameter) subtidal asteroid, *Solaster stimpsoni,* to *O. borealis.* The starfish, which was at least two orders of magnitude larger than the snail, responded by curling its ray to the aboral side of the body. Repugnatorial gland secretion applied with a pipette to the ray of *L. hexactis* elicited the same response as a living *O. borealis.*

Experiments on the Leptasterias/Onchidella *interaction*

When *L. hexactis* were offered a choice of molluscan food items from the intertidal zone (*O. borealis, Collisella digitalis, Notoacmaea scutum*), they demonstrated a predictable hierarchy of preferences in both laboratory and field trials (Fig. 4). *Onchidella borealis* were never consumed. The most frequently consumed prey was the

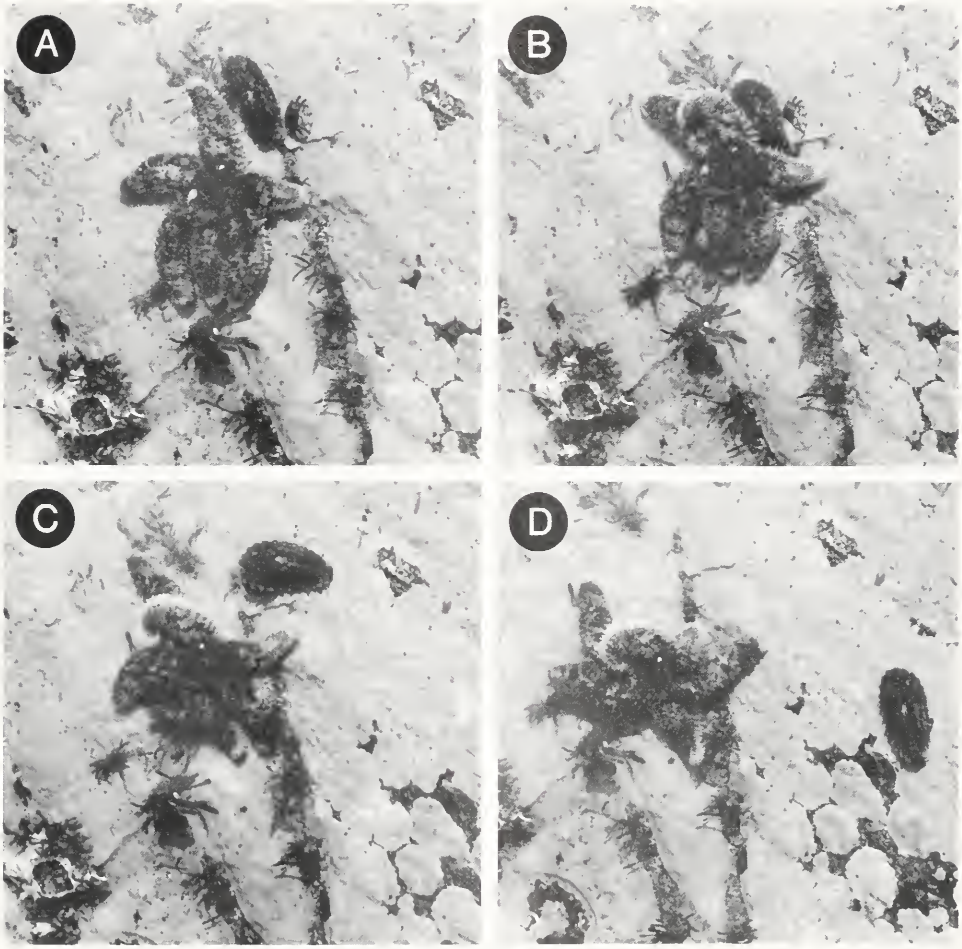

FIGURE 3. Staged encounter between *Onchidella borealis* and *Leptasterias hexactis* in a tidepool. A: Starfish touches *O. borealis* with tube feet; repugnatorial glands fire. B: Starfish curls arm aborally in response to secretions. C: *O. borealis* reverses direction of crawling while starfish remains stationary. D: *L. hexactis* continues to hold arm off substratum.

high intertidal limpet *C. digitalis. Notoacmaea scutum,* a limpet that occupies the same level of the intertidal zone as *L. hexactis* and *O. borealis,* was never consumed in laboratory experiments, but several individuals were taken during the neap tide period in the field experiments, possibly after the *C. digitalis* had all been consumed.

In the laboratory, there was a significant difference in the number of *L. hexactis* specimens remaining within holdfasts in the presence and absence of *O. borealis* (Fig. 5). Where *O. borealis* were present, the majority of starfish abandoned the holdfasts within the 2-h experimental period. Field experiments produced essentially the same result, except that the overall number of starfish remaining was lower than in the laboratory (Fig. 5).

DISCUSSION

Although interactions between molluscs and their potential predators have been studied extensively (for reviews see: Thompson, 1960; Edmunds, 1966; Todd, 1981;

TABLE III

Responses of three asteroid species to live and dead Onchidella borealis

Predator species	Condition of prey	Response				
		Aboral curl	Arm lift	Lateral movement	Combination	None
Pisaster ochraceous	live	1	26	15	5	3
	dead	0	0	2	0	48
Leptasterias hexactis	live	5	30	7	6	2
	dead	0	0	9	0	41
Henricia leviuscula	live	0	15	11	3	21
	dead	0	0	0	0	0

Responses are broken down into four categories. For the analysis in Table IV, all responses are pooled.

Faulkner and Ghiselin, 1983), these data are the first in which predator-prey interactions have been documented for a large number of potential predators of intertidal Onchidiids. In addition, our data indicate that the defensive secretion of *Onchidella borealis* has an effect on the distribution of the predatory seastar *Leptasterias hexactis.* The results indicate that *O. borealis* does not fire its repugnatorial glands in response to all potential predators, nor do all potential predators demonstrate flight behaviors in response to the glandular secretions of *O. borealis.* The most dramatic responses are found in intertidal asteroids that occupy the same approximate intertidal zone as *O. borealis* and consume small molluscs (primarily limpets) as a major portion of their diets.

Onchidella borealis did not fire their repugnatorial glands when encountering large polyclad turbellarians (*Notoplana atomata*) or when covered in flatworm mucus, even though the worms may be predators on *O. borealis* (E. P. M. Yau, pers. comm.). *Notoplana atomata* are sensitive to repugnatorial gland sections, but only on the dorsal side; the thick ventral mucus seems to effectively block the secretions in natural encounters. It may be significant that the only place in Barkley Sound where we have found *Notoplana atomata* abundant is in the same inlet and intertidal zone where the highest density of *O. borealis* is found. Additional work on the diet of the worm is needed.

The nemertean *Paranemertes peregrina* is sensitive to repugnatorial gland secretions only in the head region, even though the entire body of the worm stimulates firing of the repugnatorial glands. In the encounters we observed, the nemertean

TABLE IV

Log-likelihood analysis of the responses of three asteroid species to live and dead Onchidella borealis

Source of variation	d.f.	G	*P*
Species × prey condition	4	24.656	0.05
Species × response	4	41.717	0.01
Prey condition × response	3	218.066	0.001
Species × prey condition × response	2	1.015	ns

Raw data (Table III) are pooled into two response categories (response, no response) for the analysis.

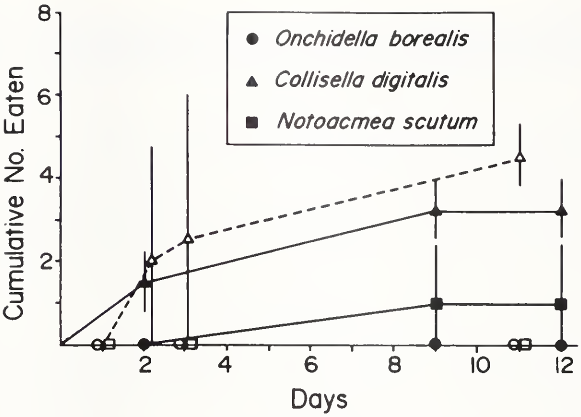

FIGURE 4. Cumulative number of *Onchidella borealis, Collisella digitalis,* and *Notoacmaea scutum* consumed by *Leptasterias hexactis* in laboratory (solid lines and points) and field (broken lines and open points) experiments in which prey were replaced as eaten. Error bars are standard deviations. In lab trials (n = 2), each bowl contained 10 individuals of each potential prey species. Field cages (n = 2) each contained three individuals of each prey species.

never attacked *O. borealis* and seldom changed the direction of its movement in response to the snails. Predatory shelled snails, *Searlesia dira* and *Thais lamellosa,* showed no response to *Onchidella borealis.*

Onchidella borealis were apparently paltatable to crabs (*Hemigrapsus nudus*); the crabs consumed dead snails readily in laboratory experiments. Live *O. borealis* were consumed less frequently, suggesting that the repugnatorial glands may reduce the predation pressure by crabs. However, our experiments with the crabs do not distinguish between the actual effects of the repugnatorial glands and other factors associated with live animals such as crawling behaviors, actively modified distributions,

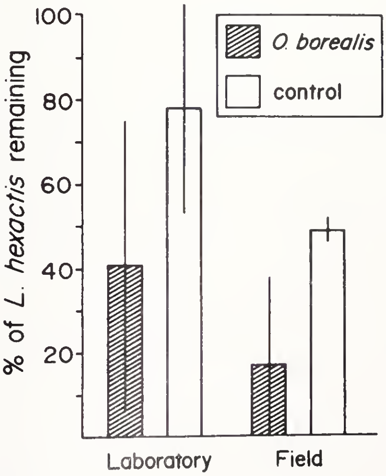

FIGURE 5. Percentage of *Leptasterias hexactis* remaining in holdfasts containing *Onchidella borealis* and no *O. borealis* in the laboratory and the field. Error bars are standard deviations.

etc. Fishes did not consume *O. borealis* in laboratory experiments, though it is not clear if this was due to the defensive secretions of the snails, to the unnatural laboratory setting, or to the food preferences of the fishes.

Effects of O. borealis *on the distribution of* L. hexactis

The effects of gastropod defense systems on the ecology of the potential prey species have been tested in a number of studies. Schmitt (1982) considered how different defense mechanisms in a guild of mesogastropods influenced distributional patterns and population dynamics of snails. Margolin (1964) studied the flight behaviors displayed by intertidal limpets upon encountering sea stars. He showed that species with effective defenses (*e.g., Notoacmaea scutum*) overlapped in their distribution with potential predators such as *L. hexactis,* while limpets without flight behaviors (*e.g., Collisella digitalis*) occupied a level of the intertidal higher than the predators. We found the same thing in this study. Like *N. scutum, O. borealis* are defended well against *L. hexactis* and overlap the predator in its distribution.

Rice (1985) has shown that a defensive mucus of the marine pulmonate *Trimusculus reticulatus* protects the snail from predation by the asteroid *Pisaster ochraceous.* However, Rice (1985) reports that, in the field, these two species commonly overlap in their distribution and can be found together in the same crevices. Conversely, our experiments suggest that although the distribution of *O. borealis* is not limited by predators, the small-scale distribution of the predatory asteroid *Leptasterias hexactis* may be influenced by high density populations of *O. borealis.* At some sites, the density of *L. hexactis* was highest just below the level of peak *O. borealis* density, but elsewhere, the two populations peaked at the same level (Eagle Pt.) or were juxtaposed (*e.g.,* Seppings Island). Thus, the upper limit of *L. hexactis* appears not to be set by *O. borealis.* The definitive experiment would be to remove all *O. borealis* from a stretch of shoreline, then note any changes in the vertical distribution of *L. hexactis.*

Both *O. borealis* and small *L. hexactis* appear to use the holdfasts of *Hedophyllum sessile* as refuges. Onchidiids are readily knocked off the substratum by waves (Arey and Crozier, 1921) so the holdfasts probably serve as buffers from wave shock. Water motion is probably less critical for *L. hexactis,* as they cling securely to rocks and feed actively at high tide (Menge, 1972). Only small starfish are found within holdfasts of *H. sessile* and *Nereocystis luetkeana* specimens, suggesting that refuges from some unidentified source of mortality (perhaps desiccation or predators) are important to juvenile *L. hexactis.*

We have shown experimentally that specimens of *Onchidella borealis* have a negative effect on young individuals of *Leptasterias hexactis* within the holdfasts, probably because the latter are expelled by repugnatorial gland secretions. Although *L. hexactis* individuals leave holdfasts to which *O. borealis* have been added, it is likely that in the field, *L. hexactis* individuals never enter holdfasts occupied by *O. borealis* specimens. It would be interesting to determine if *O. borealis* returns to the same holdfast repeatedly, just as congeners home to the same crevice (McFarlane, 1980). If so, a given holdfast may be occupied exclusively by *O. borealis* for very long periods of time. Scarcity of unoccupied holdfasts in areas of high *O. borealis* density may increase mortality of *L. hexactis* juveniles or force them to occupy other refuges such as holdfasts lower in the intertidal.

Additional field work is needed to consider the possible effect of *O. borealis* on aspects of *L. hexactis* ecology. *Leptasterias hexactis* individuals use crevices for brooding their young (Chia, 1966; Menge, 1974) and as a refuge from competition with the larger forcipulate *Pisaster ochraceous* (Menge, 1974). Large numbers of *O.*

borealis occupying crevices could limit the amount of space available to *L. hexactis,* thus forcing the small starfish into more competitive encounters with *P. ochraceous.* Such encounters have been shown to decrease the individual fitness of *L. hexactis* (Menge and Menge, 1974).

The evolutionary origin of the repugnatorial glands in *Onchidella borealis* is unknown, though it seem likely that they originated as defensive structures. At present, they may function not only in defense, but as competitive "equalizers" allowing *O. borealis* to compete successfully for a limited microhabitat in the crowded intertidal zone.

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INHIBITION OF NEMATOCYST DISCHARGE DURING FEEDING IN THE COLONIAL HYDROID *HALOCORDYLE DISTICHA* (= *PENNARIA TIARELLA*): THE ROLE OF PREVIOUS PREY-KILLING

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ABSTRACT

The colonial hydroid *Halocordyle disticha* (= *Pennaria tiarella*) kills prey with stenotele nematocysts. These nematocysts become less responsive after heavy feeding, but their killing activity is restored after hydranths are placed in fresh medium. Nematocysts of unfed hydranths became inactive when neighboring hydranths (either attached or separated) were fed; the effect increased with the amount of previous prey-killing. The loss of nematocyst activity was not due to stenotele depletion or compounds in prey fluids; waste products produced by the hydroid or prey had little effect. Extracts of capitate tentacles produced inactivation, and stenoteles were completely inhibited in a concentrated solution of stenotele discharge products. Our results indicate that the inactivation of stenoteles is due to the accumulation of materials released from the stenoteles during discharge.

INTRODUCTION

Very little is understood about the mechanisms which control nematocyst discharge in cnidarians. These intracellular secretion products ("organoids") classically have been considered to be independent effectors, acting as both receptor and effector without input from conducting pathways. More recent studies indicate some control over nematocyst discharge, as it may be influenced by the physiological state of the animal (reviewed in Mariscal, 1974). There is ample evidence for neuro-nematocyst junctions (Westfall, 1969; Westfall *et al.*, 1971), and non-neural epithelial conduction (Mackie, 1970) may also function in controlling discharge.

Repeated observations that satiated animals fail to capture or even reject offered food (Hyman, 1940; Kanaev, 1952; Mariscal, 1974) provide evidence for host control over nematocyst function. These studies have been performed extensively with *Hydra*. Burnett *et al.* (1960) suggested that the stimulation of gastrovascular stretch receptors in well-fed animals mediated stenotele inactivation, while Smith *et al.* (1974) presented evidence that perception of prey metabolites by gut cells was the sensory clue for nematocyst inhibition. More recently Ruch and Cook (1984) demonstrated that inactivation following feeding could occur without food in the gut, and that soluble factors released by the hydra during feeding served to inactivate stenoteles. The latter conclusion was based on the finding that feeding by a parent hydranth reduced the stenotele activity of nearby buds which had no gut connections with the parents, even when those buds had recently detached.

This observation raises the question of nematocyst inactivation during feeding

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by colonial cnidarians. We chose the colonial athecate hydroid *Halocordyle disticha* (= *Pennaria tiarella;* Calder and Hester, 1978) for study, as it is readily available and much is known about its feeding biology (Pardy and Lenhoff, 1968). Our research was designed to investigate three questions: (1) does stenotele inactivation occur during feeding by this hydroid? (2) How does feeding by one hydranth affect nematocyst function in neighboring hydranths? (3) What is the source of any inactivating factors?

MATERIALS AND METHODS

Colonies of *Halocordyle disticha* (= *Pennaria tiarella*) were collected from Coney Island, Bermuda, or Morehead City, North Carolina. They were maintained in running seawater at 23–27°C in nine-inch culture dishes, and fed *Artemia salina* nauplii twice daily until the day prior to use in experiments. At this time they were placed in dishes containing aerated, filtered (0.45 μm) seawater. For feeding experiments, groups of three hydranths from the same colony (either connected by the stolon or separated) were placed in small plastic dishes with 0.15 ml of seawater. In one experiment the volume was increased to 150 ml to study the influence of the volume of fluid on nematocyst activity.

Feeding procedure and assay of killing response

All responses of *H. disticha* were observed under a dissecting microscope at 30×; in some experiments a video camera fitted to a Wild M420 Macroscope was used to record observations. We used two methods to quantitatively present *Artemia* larvae to the hydranths. In the first, "planned random feeding," nauplii were drawn into a capillary tube (I. D. 0.9 mm) fitted to a mouth pipette and the total counted under a dissecting microscope. These were fed to the hydranths, and additional *Artemia* were added if all of the original allotment had been killed. Any nauplii which were still alive after inactivation of all three hydranths (see below) were subtracted from the total presented to yield the number which had been killed. In the second method, "selective feeding," nauplii were individually presented to hydranths with the tip of a flame-drawn capillary tube. Care was taken to avoid letting any prey escape and that only particular hydranths were fed at any one time.

In both procedures, we considered the nematocysts of a hydranth to be inactive when five consecutive contacts with larvae produced neither killing nor capture (adherence to tentacles; Ruch and Cook, 1984). Typically the time required to feed control hydranths to inactivation was less than 40 minutes.

Selective feeding of connected and separated hydranths

To determine if feeding by neighboring hydranths affected the nematocyst activity of a hydranth, we performed experiments involving hydranths connected by a stolon, and hydranths which had been separated by cutting the stolon. The separated hydranths were allowed 6 h to heal and then were placed 2 mm apart in a dish; this spacing corresponded to their usual positions in an intact colony. In each case successive polyps were "selectively" fed to inactivation. After inactivation the connected hydranths were separated, and all of the hydranths transferred to individual dishes with fresh filtered seawater. They were then tested for the recovery of feeding ability.

Determination of stenotele complements in fed and unfed hydranths

Stenotele numbers in 24-hour starved hydranths and those which had been fed until nematocysts no longer functioned were compared in cell maceration preparations (David, 1973). Individual hydranths were separated, and the number of large (6.8 μm) stenoteles in capitate tentacles were counted using phase contrast microscopy (400×).

Media exchange experiments

To determine if factors released from either prey or from hydranths were effective in producing nematocyst inactivation, we determined the killing responses of hydranths which were fed in solutions in which other hydranths had fed. Each original group of three hydranths was fed to inactivation by the "planned random feeding" technique. The solution was drawn off with a fine-tipped micropipette, free of dead *Artemia* or other debris, and placed in a clean container. A fresh branch with three hydranths was then placed in this "used" solution and the hydranths were fed in the same manner. The number of *Artemia* killed at inactivation was then compared in both groups.

The effect of prey homogenate on stenotele activity

In an attempt to determine if prey fluids could be the source of any inactivating factor, hydranths were fed in a prey homogenate solution. Two hundred *Artemia* nauplii were homogenized in 0.5 ml filtered seawater with a hand grinder. To maximize cell disruption, the solution was electrified with platinum electrodes attached to a Grass S6 stimulator set at 80 V for 3 s. This solution was then filtered (0.45 μm), and a branch with three hydranths was fed by "planned random feeding" in 0.15 ml of the filtrate. Each test solution thus contained the extract of approximately 70 nauplii. Control hydranths were fed in electrified filtered seawater.

Feeding in capitate tentacle solution

We investigated the possibility that capitate tentacles were the source of inactivating factors by feeding hydranths in extracts of capitate tentacles. Two hundred capitate tentacles were homogenized in 0.5 ml of filtered seawater; each 0.15 ml feeding volume thus contained the extract of approximately 60 tentacles. The homogenate was electrified as above to induce stenotele discharge (see Results), and the resultant solution was filtered. As a control the internodal tissue was extracted from five 4-cm sections of stolon. This tissue, which is not involved with prey-killing, was treated in the same manner as the tentacles. Groups of three connected hydranths were then fed by "planned random feeding" in each solution.

Feeding in purified nematocyst solution

"Planned random feeding" of hydranths also occurred in an extract of purified nematocysts. Nematocysts were obtained from *H. disticha* using a modification (P. Suchy, in prep.) of the technique of Lane and Dodge (1959). Perisarc-free hydranths were gently stirred with a magnetic stirrer in 10 volumes of distilled water for 24 h at 4°C to autolyse tissue. The resulting suspension was screened through bolting silk and 35 μm and 28 μm Nytex screening to remove large particulates and centrifuged at high speed with an IEC benchtop centrifuge. The sedimented material was resuspended twice in 10 m*M* MOPS adjusted to pH 7.4 with Trizma base and centrifuged at high speed for 5 min. After this procedure, microscopic examination revealed that 97% of the particulate material consisted of nematocysts, the rest being unidentifiable cellular debris. These preparations were stored frozen with the addition of 30 μg/ml of aprotinin (Sigma) as a protease inhibitor.

The final concentration of nematocysts was determined with a hemocytometer to be 85,000 per ml. The thawed solution was centrifuged at high speed in an IEC clinical centrifuge for 2 min, and the nematocysts were resuspended in filtered seawater. The solution was then electrified at 80 V for 3 s to induce stenotele discharge, and the solution passed through a 0.45 μm filter to remove particulate material. Thus the volume used in our feeding experiments (0.15 ml) contained the discharge products of approximately 12,000 stenoteles (20 times the stenotele complement of a single hydranth; Table I). Three connected hydranths were placed in this solution, while the control group was placed in electrified filtered seawater. Both groups were then tested by "planned random feeding."

RESULTS

The effect of feeding by neighboring hydranths on nematocyst function

We studied the effect of feeding by neighboring hydranths on prey-killing by selectively feeding each of three connected hydranths until prey-killing ceased. The results of feeding the terminal hydranth first, followed by feeding the middle and then the innermost hydranth, are shown in Figure 1a. The middle hydranths showed a 46.6% decrease in activity relative to the first-fed hydranth, while those which were fed last showed an 83.8% decrease. The correlation between the number of nauplii killed and the order of feeding was highly significant ($r = -0.967$, $P < 0.01$; $n = 18$), showing clearly that feeding by hydranths reduced the nematocyst activity of neighboring polyps, and that the effect appears to be cumulative.

In other experiments we varied the order by feeding first the middle hydranth with virtually identical results (Fig. 1b). The hydranths which were fed second showed a decrease of 51.2%, while those which were fed last showed an 84.8% decrease. The correlation was the same in as the previous experiment ($r = -0.967$, $P < 0.01$; $n = 9$).

To determine if inactivation was due to information passed between the hydranths by neural elements, by other cell-cell contacts, or via the gastrovascular cavity we repeated the experiment with groups of three hydranths which had been dissected apart. Again, there was a decrease in nematocyst function with increased feeding. Relative to the initial value, the killing response of the hydranth which was fed second decreased by 58.6%, while the hydranth which fed last showed a 92.1% decrease (Fig. 2). The correlation between the killing response and the order of feeding was again highly significant ($r = -0.944$, $P < 0.01$; $n = 9$). This result demonstrates that decreased killing occurs without cellular interactions between polyps. Clearly, neither nervous inputs from prior fed hydranths nor food ingested by these hydranths are required for stenotele/cnidocyte inactivation by affected hydranths.

Figure 3 summarizes the effect of prior feeding on the killing response. This figure contains the combined data (connected and separated hydranths) for the second- and third-fed hydranths of Figures 1 and 2. The percentage of inhibition of killing (relative to the first-fed hydranth) is plotted against the number of shrimp which were killed in the solution prior to testing. The significant negative correlation ($r = -0.694$, $P < 0.01$) demonstrates that prior feeding by hydranths of *H. disticha* reduces the killing response of neighboring polyps, and indicates that the effect is additive, possibly due to the accumulation of factors in the feeding environment.

"Recovery" experiments confirmed the role of accumulating factors in stenotele

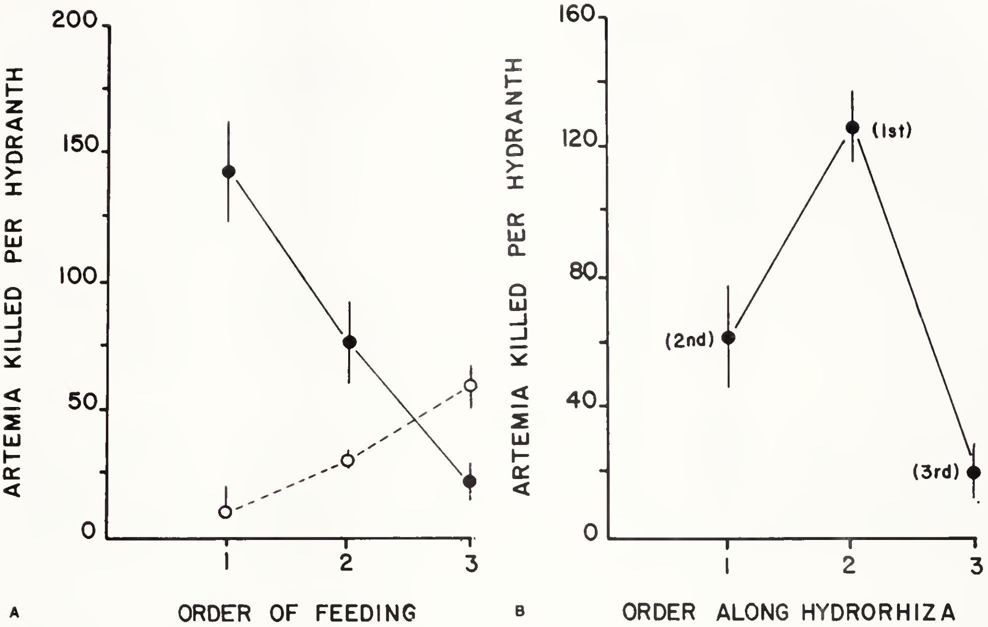

FIGURE 1. Stenotele activity during selective feeding of connected neighboring hydranths of *H. disticha.* Vertical bars = ±1 standard deviation. A. Selective feeding of hydranths in order along branch. ●——●, stenotele inactivation during feeding; ○---○, recovery following transfer to fresh medium. n = 6 for each point; r = −0.967. Variation in feeding order: middle hydranth fed first, then left, then right; order of feeding is indicated. n = 3 for each point.

inhibition. Branches of three connected hydranths were fed to inactivation, then rinsed in filtered seawater and dissected apart. The separated hydranths were then retested for their killing ability in individual dishes. The hydranths which had been fed first, and which originally had killed the most nauplii killed an additional 10.8 ± 10.0 shrimp (n = 3). The middle hydranths showed an intermediate killing response ($\bar{X}$ = 30.7), while the last-fed hydranths (which had killed very few *Artemia*) killed an average of 59.3 ± 7.5 shrimp. Thus, all three groups showed a reversal of the inactivation upon return to fresh seawater, consistent with the accumulation of inactivating factors in the original feeding solution. However, neither the second nor the third hydranths in each series killed as many total *Artemia* as the original number killed by first-fed hydranths.

Stenotele complements of fed and unfed hydranths

The effects of feeding neighboring polyps on nematocyst function indicated that the loss of nematocyst function with feeding accrued from the inactivation of nematocysts, rather than their depletion during feeding. To examine this possibility, we counted stenoteles in the capitate tentacles of groups of three connected hydranths, fed selectively as above. Twenty-four-hour starved hydranths served as controls.

The stenoteles of capitate tentacles could be readily distinguished from those of the filiform tentacles by their location and greater size (6.8 μm *vs.* 4.5 μm). We used these because they were more conspicuous, and were conveniently fewer in number. We did not distinguish between discharged and undischarged stenoteles in our

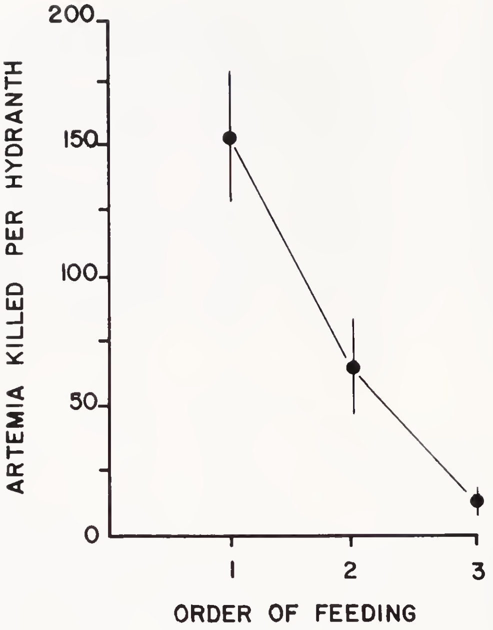

FIGURE 2. Inactivation of stenoteles during feeding of separated hydranths of *H. disticha*. Each hydranth selectively fed to inactivation before the next hydranth was tested. Order of feeding as in Figure 1a. n = 3 for each point; r = −0.944.

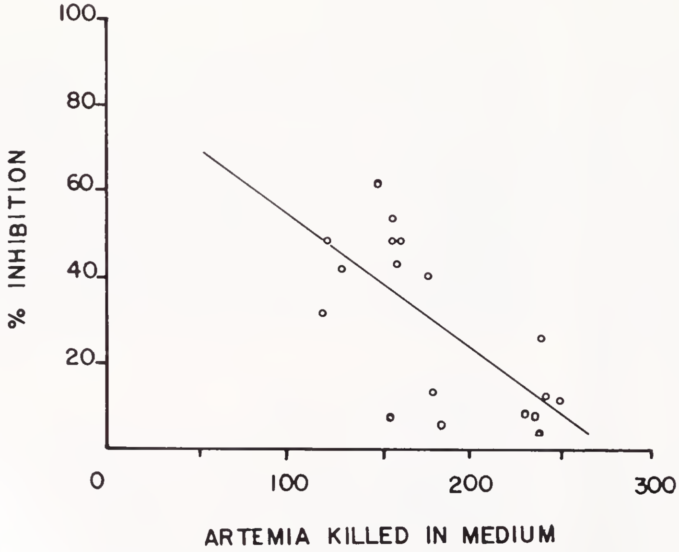

FIGURE 3. The effects of prior feeding on the killing responses of the second- and third-fed hydranths. The inhibition of killing is expressed as the percentage of *Artemia* nauplii killed by first-fed hydranths. The regression line is significant at the 1% level. (Data of Figs. 1 and 2.)

TABLE I

Complements of large (6.8 μm) stenoteles in fed and unfed hydranths of Halocordyle disticha *determined from counts of cell maceration preparations*

Feeding regime	Stenoteles per hydranth ($\bar{X}$ ± S.D.)
First-fed	98 ± 20.5 ($P < 0.001$)*
Second-fed	283 ± 40.3 ($P < 0.001$)*
Third-fed	706 ± 45.1 (n. s.; $P > 0.3$)*
24 h starved	737 ± 29.1

* Probability that mean is equal to control value; one-way ANOVA.
The sequence of feeding of neighboring hydranths corresponds to that in Figure 1.
n = 3 for all cases.

counts. Unfed hydranths possessed 737 ± 29.1 (n = 3) stenoteles. The last-fed hydranths, which showed the greatest decrease in stenotele activity, possessed 706 ± 45.1 (n = 3); thus, they had become inactivated with essentially a full complement of stenoteles. The hydranths which were fed second had 283 ± 40.9, while those which had been fed first retained only 98 ± 70.5 stenoteles (Table I).

Media exchange experiments

We tested the hypothesis that the accumulation of soluble factors in the feeding medium inactivated stenoteles of *H. disticha* by feeding starved hydranths in the media in which hydranths had previously been inactivated (Table II). The hydranths fed in "conditioned" media exhibited a greatly reduced killing response ($\bar{X}$ = 78.1% decrease; Table II). Interestingly, in a second experiment the control (original) hydranths killed more *Artemia* than did the controls of the first experiment, and the hydranths fed in this "used" solution were more inactivated ($\bar{X}$ = 94.5% decrease). This suggested a "dose-dependent" response, as if the inactivation were a function of the number of shrimp which were killed.

Volume dependency of inactivation

To investigate further the dose-dependent nature of stenotele inactivation during feeding, hydranths were tested in 150 ml of seawater, rather than the usual test volume of 0.15 ml. This would represent a thousand-fold dilution of any soluble factors.

TABLE II

Effects of feeding Halocordyle disticha *in seawater in which other hydranths had previously fed*

Experiment	Group	# of nauplii killed	% Decrease
1	Control	160	78.1%
	Experimental	35	
2	Control	261	95.4%
	Experimental	12	

The control group represents the first-fed group; experimental hydranths were fed in the solutions in which these had fed. Test solutions were filtered (0.45 μm) prior to use.
n = 2.

TABLE III

The effects of extracts of captitate tentacles and internodal tissue on stenotele function in Halocordyle disticha

Group	# of nauplii killed (±S.D.)
Fed in tentacle extract	0.0
Fed in internodal extract	192 ± 7.0
Controls	202 ± 12.0

Tissue extracts were prepared by electrifying tissue homogenates; controls were tested in electrified seawater.

Branches with three connected hydranths were selectively fed to inactivation. As in the previous experiments, the hydranth which was fed first killed the greatest number of *Artemia* ($\bar{X}$ = 237.5 ± 14.8; n = 2), the second-fed hydranth had an intermediate response ($\bar{X}$ = 117 ± 12.7; n = 2), and the third hydranth killed relatively few in comparison ($\bar{X}$ = 57.5 ± 7.8). The recovery of killing ability after transfer to fresh seawater was tested in these also. As in the connected hydranth experiment, the hydranth which had been fed first killed relatively few additional *Artemia* (20 ± 12.7; n = 2), the second-fed hydranth killed an intermediate number (54 ± 17; n = 2), and the hydranth fed third was relatively active (76 ± 8.5; n = 2). In all cases, the absolute numbers of shrimp killed by all three groups was somewhat higher than those which were tested in the smaller volume (see connected and separated experiments).

Feeding in prey homogenate

We examined the possibility that prey tissue might be the source of stenotele inactivating factors by feeding a branch with three hydranths in a solution containing the cell-free extract of 200 *Artemia* nauplii. This is approximately the number of *Artemia* killed by control hydranths throughout this study. Hydranths fed in electrified seawater alone killed 199.3 ± 4.0 nauplii (n = 4), while the experimental group killed on average 50 additional *Artemia* (249.3 ± 10.3; n = 4). This significant difference ($P < 0.01$; *t*-test) shows that prey homogenate stimulates the killing response. Thus inactivation cannot be due to factors in prey fluids which might be released during feeding.

The effects of feeding in a cell-free extract of capitate tentacles

An extract of capitate tentacles (see Materials and Methods) was used to determine if the tentacles were a source of inactivating factors. One control group was fed in electrified seawater, while a second control group was tested in an electrified extract of internodal tissue. While we had thought that the internodal tissue was nematocyst-poor, microscopic inspection of this tissue revealed that it contained many small nematocysts which could not be classified even with transmission electron microscopy (Clark, unpub.). Examination of electrified internodal tissue with light microscopy showed that none of these nematocysts were discharged. By contrast, 85–90% of the stenoteles of capitate tentacles were discharged by our electrification procedure.

Both control groups exhibited normal kill responses (Table III), indicating that neither metal-poisoning by platinum electrodes nor material from nematocyst-poor tissue had much effect on stenotele behavior. We observed no killing by any hydranths which were fed in the tentacle extract solution (n = 18; Table III). When the inactivated hydranths from this experiment were transferred to fresh seawater, both control groups exhibited greater recovery ($\bar{X} = 43.3 \pm 5.3$ additional nauplii killed) than the experimental hydranths. These killed only 13 ± 5.3 nauplii, even though they had killed no prey during the experiment and presumably contained a full complement of stenoteles.

The effects of nematocyst discharge solution

The previous observations strongly suggested that stenoteles from capitate tentacles were the source of the inactivating factor. To directly test this possibility, we fed branches in a filtered solution of nematocyst discharge products (approximately 12,000 stenoteles in 0.15 ml), while controls were fed in electrified seawater. In three replicates, none of the experimental hydranths killed or captured any prey; controls exhibited a normal kill response ($\bar{X} = 183 \pm 19.7$ nauplii killed; $n = 12$). When the experimental hydranths were rinsed and placed in fresh seawater, they killed an average of four nauplii.

This was the lowest degree of stenotele recovery which we observed in any of our experiments. Both the complete inhibition of nematocyst activity and the low recovery represent exaggerated responses to the contents of approximately 20 times the number of stenoteles used during normal prey-killing (Table I).

The effects of hydroid or prey metabolites on the killing response

The waste products of hydroids or *Artemia* nauplii, produced under the conditions of our experiments, are possible sources of inactivating substances. We examined this possibility in two ways. In one experiment we kept branches with three hydranths in 0.15 ml of filtered seawater under microscope illumination for 1–3 h before feeding a single hydranth. All hydranths continued to kill nauplii, even after 3½h of these conditions. Each of the four hydranths tested killed at least 60 nauplii, and none were inactivated when feeding ended. In a second experiment, we kept a dense suspension of *Artemia* nauplii (*ca.* 220 nauplii per 0.15 ml) at room temperature for 1 h before the solution was filtered through bolting silk, and a single polyp on a three polyp branch selectively fed in 0.15 ml of the filtrate. In two trials inactivation was not achieved after 65 and 70 nauplii were killed; we did not present additional prey.

While these observations do not rule out inactivating effects of accumulated waste products such as CO_2, we feel that they are unlikely to be major factors for two reasons. First, our feeding experiments would be typically completed in less than an hour; second, the magnitude of inactivation we found in other experiments, particularly in third-fed hydranths and those treated with stenotele extracts, was much greater than what accumulated wastes could produce.

DISCUSSION

Feeding responses and the kill response in H. disticha

Pardy and Lenhoff (1968) described prey killing in *Halocordyle disticha* (= *Pennaria tiarella*). Prey are immobilized by numerous small stenoteles of the filiform tentacles. This initiates the feeding responses which consist of the bending of the oral cone and the opening of the mouth. Then prey are actually killed by the large stenoteles of the capitate tentacles prior to engulfment of the prey by the mouth. "Feeding response" refers to the behavioral pattern which occurs after capture and before ingestion of prey. Pardy and Lenhoff (1968) demonstrated that this behavior is a response to free proline which is released in prey fluids following penetration of the body wall by stenoteles. While we often observed these responses, we were only concerned with the discharge of stenotele nematocysts (kill response) of *H. disticha* hydranths during feeding. Thus our findings on nematocyst inactivation do not contradict other studies (*e.g.*, Burnett *et al.*, 1968), which propose that nematocyst discharge in hydra may stimulate feeding responses.

Inactivation of nematocysts/cnidocytes during feeding in H. disticha

Our study shows that the prey-killing stenoteles of the colonial hydroid *H. disticha* are inactivated during periods of heavy feeding, although we cannot say if it is the responsiveness of cnidocyte or the nematocyst itself which is affected. Nematocyst inhibition following feeding has previously been reported in *Hydra* (Burnett *et al.*, 1960; Smith *et al.*, 1974; Ruch and Cook, 1984) and other cnidarians (Sandberg *et al.*, 1971; Mariscal, 1973). The reduction in nematocyst activity during feeding may be a general phenomenon among cnidarians which conserves these complex structures (Mariscal, 1974).

Previous workers had proposed that nematocyst inactivation during feeding by *Hydra* resulted either from the distention of endodermal stretch receptors following ingestion (Burnett *et al.*, 1960), or the reception of prey metabolites by gut cells (Smith *et al.*, 1974). Ruch and Cook (1984) showed that nematocyst/cnidocyte inhibition occurred in "gutless" hydra, without either of these sensory inputs. They ascribed inhibition to the accumulation of soluble factors released from a feeding hydra (Ruch and Cook, 1984). Our experiments involving media exchanges (Table II), the selective feeding of separated hydranths (Fig. 1), and recovery of stenotele activity are all consistent with the notion that this occurs in *H. disticha*. The effect is cumulative, being greater when more prey are killed (*cf.* the second- and third-fed hydranths in Fig. 1, and the media exchange experiments). Prey fluids are clearly not involved, as we found that prey extract stimulated stenotele discharge (*cf.* Ruch and Cook, 1984). Pardy and Lenhoff (1968) reported that proline (the "feeding activator" for *H. disticha*, doubtless present in prey fluids) elevated cnidocils on capitate tentacles of *H. disticha*. Their observation indicates increased responsiveness of cnidocytes following exposure to prey fluids. Our experiments also show that waste metabolites of the hydroids and the prey are unlikely to have a major role in inactivation.

Aside from ingestion and the presence of prey fluids, the most obvious difference between a feeding and non-feeding hydranth is the number of fired nematocysts. Our experiments provide evidence these fired nematocysts are a source of inactivating material. Both extracts of capitate tentacles and the discharge products of purified stenoteles produced complete inhibition. The results of the capitate tentacle extracts are particularly relevant, as these preparations contained more realistic concentrations of nematocyst discharge products (roughly those from 1–2 hydranths). Significantly, the extract of internodal tissue had no effect, even though it contained many small, and probably immature, nematocysts. These nematocysts were disrupted by our homogenization procedure, and the intracapsular material would have been present in the test solution. The implication is that discharge products, rather than intracapsular or non-nematocyst material, are responsible for inhibition. The preparation of purified stenoteles contained an unnaturally high concentration of discharge products, and the results of this experiment are an extreme case. Both of these experiments strongly suggest that mature, fired stenoteles are the source of inactivating substances, the most likely of which are the toxins themselves. The identity of these compounds and how they are sensed are unknown, and we cannot say if they affect the cnidocyte or the nematocyst itself. Perhaps the cnidocil apparatus or other receptors on the ectodermal surface could be involved.

The "community" effect between hydranths in a colonial hydroid

Selective feeding of three hydranths revealed a community effect, in that feeding by one hydranth reduced the number of nauplii killed by neighboring hydranths in the same medium when they still contained functional stenoteles. Third-fed hydranths, which killed very few nauplii, were inactivated with virtually a full complement. This "community effect" is similar to the inactivation of attached buds due to parental feeding in hydra (Ruch and Cook, 1984) and of one head of a two-headed graft caused by the feeding of the other head (Smith *et al.*, 1974). As the products of ingestion by single polyps of colonial cnidarians can be transported to other areas of a colony (*H. disticha*, Rees *et al.*, 1970; gorgonians, Murdock, 1978; see also Gladfelter, 1983), inactivated hydranths would still receive the benefits of feeding by the original polyp. *H. disticha* is particularly interesting, given the possibility that non-feeding hydranths receive more from fed polyps than do feeding hydranths (Rees *et al.*, 1970).

Our results may reflect artifacts of feeding in small volumes and under static conditions. To what extent would such inactivation occur under natural conditions? Inactivation of stenoteles occurred both in 0.15 ml and 150 ml test volumes. The lack of an evident dilution effect suggests the chemical "halo" zone which Loomis (1961) postulated to exist around a hydra, or the "ultramicroenvironment" within 20 nm of the ectodermal surface where charged surface molecules could influence the concentration and binding of molecules to the surface (Lenhoff, 1965). Ruch and Cook (1984) found similar effects of dilution on nematocyst inactivation in *Hydra.* Possibly the boundary layers which exist around polyps when exposed to flow situations in nature (see Patterson, 1984, and references therein) would also permit the accumulation of inactivating factors in the vicinity of a heavily feeding hydranth. Whether "neighborhood inactivation" actually occurs in the field, particularly in tidal flow situations where *H. disticha* commonly occurs, is not clear. Experiments involving controlled feeding of individual hydranths of *H. disticha* would be difficult under field conditions of tidal flow.

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EFFECT OF HOST FASTING AND SUBSEQUENT REFEEDING ON THE GLYCOGEN METABOLIZING ENZYMES IN *HYMENOLEPIS DIMINUTA* (CESTODA)

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ABSTRACT

During host fasting, total glycogen synthase activity in *Hymenolepis diminuta* increased to a maximum at 48 h and decreased thereafter. The activity ratio of glycogen synthase I to total glycogen synthase (I/T), however, decreased before rising again. The increase in the active form of glycogen synthase after 48 h of host fasting might prevent complete exhaustion of glycogen during prolonged starvation. The total and active glycogen phosphorylase activities increased throughout the whole fasting period.

Twenty-four hours after refeeding the fasted host, the glycogen synthase and phosphorylase activities in *H. diminuta* decreased. *In vitro* studies, however, revealed an immediate increase in the glycogen synthase activity when glucose was available.

Protein content of worms artificially reduced in length, from normal hosts was comparable to that of the 48 h starved worms but not to their glycogen content, glycogen synthase, and phosphorylase activities.

INTRODUCTION

Glycogen, the major carbohydrate reserve, serves as an important energy source for *Hymenolepis diminuta* (Read, 1972; Mied and Beuding, 1979). Glycogen synthase (UDP-glucose:glycogen-4-glycosyl transferase EC 2.4.1.11) and glycogen phosphorylase (α-1,4-glucan-orthophosphate glucosyl transferase EC 2.4.1.1) are, therefore, the two main regulatory enzymes critical to the energy metabolism of the worm.

Effect of host fasting on the glycogen synthase activities in *H. diminuta* has been determined. Fasting the rat host for 24 h decreases the active form of the enzyme. Its activity, however, greatly increases one hour after the host is refed. (Mied, 1975; Dendinger and Roberts, 1977). However, the correlation between the activities of glycogen synthase and glycogen phosphorylase in *H. diminuta* during fasting and refeeding have not been determined. Thus the present investigation was undertaken to elucidate the regulatory mechanism of these two enzymes in *H. diminuta* in response to physiological changes of fasting and subsequent refeeding of the host.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 75–125 g were force-fed 12 cysticercoids of *Hymenolepis diminuta* reared in adult *Tenebrio* spp. Before and after infection, the rats were fed Purina Laboratory Chow and water *ad lib.* Twenty-four-day-old worms in hosts fasted for various periods were flushed from excised gut with saline solution (Ip and Fisher, 1982a) containing 120 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M*

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$MgCl_2$, 10 m*M* $NaHCO_3$, 7.5 m*M* Na_2HPO_4, and 2.5 m*M* KH_2PO_4 equilibrated to pH 7.4 with 5% carbon dioxide in nitrogen. The worms were removed from their host between 0700 and 0900 h during each experiment. The wet weight of the worms was obtained using a Sartorius Electronic Semimicro Balance 2024 MP 6. For the determination of wet weight: dry weight ratio (WW/DW), the weighed worms were dried in the oven at a temperature of 95°C for 24 h, after which the dry weight was recorded.

For the determination of glycogen and protein content and enzyme activities, the weighed worms were homogenized in an ice-cold buffer containing 50 m*M* Hepes (N-2-hydroxy-ethyl-piperazine-N-2-ethane sulfonic acid) buffer (pH 7.4), 5 m*M* ethylenediamine tetraacetic acid (EDTA), 50 m*M* NaF, and 40 m*M* 2-mercaptoethanol (βME). The samples were then centrifuged at 25,000 × *g* for 10 min at 4°C. The supernatant fluid was maintained at ice-cold conditions before various assays were performed.

Enzyme assays

Glycogen synthase. The activity of glycogen synthase was determined by measuring the incorporation of UDP-(^{14}C)glucose (Amersham) into glycogen according to Villa-Moruzzi *et al.* (1979). For total activity determination, 20 μl of the supernatant fluid was introduced into an assay mixture containing 50 m*M* Hepes (pH 7.4), 1% *H. diminuta* glycogen, 6.7 m*M* UDP-(^{14}C)glucose (0.25 μCi/μ*M*), and 10 m*M* glucose-6-phosphate in a total volume of 80 μl. For glycogen synthase I determination, glucose-6-phosphate was omitted from the assay mixture. Radioactivity was determined by using Biofluor (NEN) and a Packard Tri-Carb 300 liquid scintillation spectrometer. One unit of enzyme activity is defined as the incorporation of one μmole of (^{14}C)glucose from UDP-(^{14}C)glucose into glycogen per minute at 30°C (Donahue *et al.*, 1981).

Glycogen phosphorylase. The activity of glycogen phosphorylase was determined in the direction of glycogen synthesis (Gilboe *et al.*, 1972). For total activity determination, 20 μl of the supernatant fluid was introduced into an assay mixture containing 10 m*M* (^{14}C)glucose-1-phosphate (Amersham) (0.30 μCi/μ*M*), 1% *H. diminuta* glycogen, 25 m*M* imidazole, 1 m*M* EDTA, 40 m*M* βME (pH 6.1), and 1 m*M* 5′-AMP in a total volume of 80 μl. For phosphorylase 'a' determination, 5′-AMP was omitted from the assay mixture. One unit of enzyme activity is defined as the incorporation of one μmole of (^{14}C)glucose from (^{14}C)glucose-1-phosphate into glycogen per min at 30°C (Donahue *et al.*, 1981).

Glycogen purification

Glycogen required for the above assays was purified with methanol according to Donahue *et al.* (1981). The purity of the lyophilized glycogen was determined according to Montgomery (1957). Preparations of at least 95% purity with no indication of the presence of protein and inorganic phosphate were used.

Glycogen and protein assay

To determine the glycogen level of the tissue extracts, samples were immediately transferred into boiling water after centrifugation to stop all enzymatic reactions. The glycogen was then precipitated and purified using the method of Good *et al.* (1933) and assayed using the method described by Montgomery (1957) and Roehrig and Allred (1974) with bovine liver glycogen (Sigma Co.) as the standard.

TABLE I

Water content of H. diminuta *under different experimental conditions*

Host's condition	n	WW/DW ratio	Water content (%)
No fasting	3	4.45 ± 0.19	77.9 ± 0.8
24 h fasting	3	4.18 ± 0.12	76.4 ± 0.3
48 h fasting	2	4.61 ± 0.34	78.3 ± 1.6
72 h fasting	3	4.29 ± 0.76	72.9 ± 3.6
96 h fasting	2	4.25 ± 0.87	72.2 ± 4.1
No fasting*	4	4.51 ± 0.19	77.8 ± 0.9

* Worms from unfasted host reduced artificially to size of those obtained from 48 h fasted host.

The protein content in the extract was determined according to Bradford (1976). Standard used was bovine serum albumin (Sigma Co.).

RESULTS

There was no significant change ($P > 0.05$) in the wet weight:dry weight ratio of *H. diminuta* from unfasted as well as fasted hosts (Table I). Since the worm's water content remained relatively constant regardless of the experimental conditions applied, the protein and glycogen content as well as the enzyme activities were expressed in terms of the wet weight (WW) of the worm.

The protein contents increased significantly ($P < 0.05$) during a 96-h fast (Table II). Upon refeeding, the protein content decreased when compared to that of worms from fasted hosts (Table IV). As the protein content fluctuated with different experimental conditions, it was not suitable for use as a traditional reference to express enzyme activity.

We attempted to assay the worms' glycogen content using the method described by Roehrig and Allred (1974) which specifically measures glycogen enzymatically and avoids potential errors in extraction and subsequent precipitation. However, the above method was unsuitable as it was affected by βME present in the homogenizing buffer which is necessary to minimize phosphoglucomutase activity. Therefore, the

TABLE II

Effect of host fasting on glycogen contents, protein contents, and glycogen synthase activities of H. diminuta

Host's condition	n	No. of worms	Protein (% ww)	Glycogen (% ww)	Glycogen synthase activity* Total (T)	I	D	Ratio I/T
No fasting	5	12 ± 1	1.8 ± 0.5	3.0 ± 1.0	966 ± 45	71 ± 45	895 ± 62	0.073
18 h fasting	2	10 ± 4	2.0 ± 0.5	1.8 ± 0.5	903 ± 98	37 ± 1	866 ± 98	0.041
24 h fasting	3	10 ± 1	2.3 ± 0.9	1.6 ± 0.4	1327 ± 318	72 ± 24	1254 ± 273	0.054
48 h fasting	3	7 ± 2	3.7 ± 0.4	0.6 ± 0.4	2175 ± 529	250 ± 97	1926 ± 422	0.115
72 h fasting	2	3 ± 0	4.4 ± 0.3	0.36 ± 0.01	1752 ± 493	161 ± 77	1592 ± 416	0.092
96 h fasting	2	3 ± 2	6.9 ± 0.3	0.49 ± 0.04	1413 ± 540	333 ± 70	1081 ± 610	0.236
No fasting**	1	12	3.3	4.1	1203	33	1170	0.027

* Unit: activity per gm (ww) worm.

** Worms from unfasted host reduced artificially to size of those obtained from 48 h fasted host.

TABLE III

Effect of host fasting on glycogen phosphorylase activities of H. diminuta

		Glycogen phosphorylase activity*			
Host's condition	n	Total (T)	a	b	Ratio a/T
No fasting	5	229 ± 60	183 ± 57	46 ± 15	0.799
18 h fasting	2	175 ± 45	151 ± 44	24 ± 1	0.863
24 h fasting	3	270 ± 166	210 ± 153	59 ± 21	0.778
48 h fasting	3	311 ± 83	222 ± 59	90 ± 24	0.714
72 h fasting	2	414 ± 33	397 ± 72	59 ± 74	0.959
96 h fasting	2	630 ± 72	469 ± 21	161 ± 96	0.744
No fasting**	1	635	416	219	0.655

* Unit: activity per gm (ww) worm.

** Worms from unstarved host reduced artificially to size of those obtained from 48 h fasted host.

traditional method of glycogen extraction by Good *et al.* (1933) and Montgomery's (1957) method of glycogen analysis were chosen since the majority of the polysaccharide present in *H. diminuta* were glycogen (Read, 1972; Dendinger and Roberts, 1977, Mied and Beuding, 1979). After extraction, no significant difference was observed between the results obtained by using the methods of Montgomery (1957) and those of Roehrig and Allred (1974). The percentage recovery of the extraction procedure was estimated to be 98.12 ± 0.23 (n = 4) when such method was applied to 100 mg of standard bovine liver glycogen (Sigma Co.). The glycogen levels in *H. diminuta* decreased significantly ($P < 0.05$) during a 48-h fast and leveled off thereafter (Table II). When the host was refed, the worm's glycogen level increased significantly ($P < 0.05$) (Table IV).

Glycogen synthase and phosphorylase activity in H. diminuta *during host fasting and refeeding*

The total glycogen synthase activity of *H. diminuta* increased significantly ($P < 0.05$) during the first 48 h of fasting with a subsequent significant decrease ($P < 0.05$) at 96 h (Table II). The ratio of glycogen synthase I activity to total activity (I/T), however, decreased before rising again. When the host was refed after starvation, there were decreases in both the worm's total glycogen synthase activity and the I/T ratio (Table IV).

During fasting, the total as well as the active 'a' form of glycogen phosphorylase increased significantly ($P < 0.05$) (Table III). After refeeding, the phosphorylase activity decreased (Table V).

Effect of artificially reducing the size of H. diminuta *on its enzyme activities*

When the host was fasted, there was not only a decrease in the number of worms in the host (Table II) but also a reduction of their length. Therefore an experiment was performed to determine if the changes in enzyme activities reported above were biochemical adaptations to the starving conditions or were merely due to the uneven distribution of enzymes along the length of the parasites.

Unstarved worms were artificially shortened by cutting with a pair of scissors to the approximate length of those obtained from the 48 h starved host. Their protein

TABLE IV

Effect of in vitro *refeeding of the host after fasting on the protein contents, glycogen contents, and the glycogen synthase activities in* H. diminuta

Host's condition	No. of worms	Protein (% ww)	Glycogen (% ww)	Glycogen synthase activity*			
				Total (T)	I	D	Ratio I/T
No fasting	12	1.6	2.9	942	70	872	0.074
24 h fasting + 24 h refeeding	11	1.5	2.8	863	27	835	0.031
48 h fasting + 24 h refeeding	12	3.1	2.5	1742	65	1676	0.037
96 h fasting + 24 h refeeding	6	2.4	4.8	1198	40	1158	0.034

* Unit: Activity per gm (ww) worm.

and glycogen contents as well as glycogen synthase and phosphorylase activities were examined. The amount of protein (3.3%) present in these worms was comparable to that of the 48 h fasted ones but the glycogen content (4.1%) was significantly higher ($P < 0.05\%$) (Table II). The glycogen synthase activity in these worms was lower than these obtained from the fasted hosts while the phosphorylase activity was higher (Table II and III).

In vitro *refeeding studies on the enzyme activities*

The immediate effect of refeeding on the enzyme activities cannot be determined under *in vivo* conditions as food fed to the host requires several hours to become available to the parasites. An *in vitro* study thus was performed. Twenty-four-day-old worms from five rats fasted for 48 h were randomized and sorted into groups of seven,

TABLE V

Effect of in vitro *refeeding of host on the glycogen phosphorylase activities of* H. diminuta

Host's condition	No. of worms	Glycogen phosphorylase activity*			
		Total (T)	a	b	Ratio a/T
No fasting	9	237	135	102	0.570
24 h fasting + 24 h refeeding	11	173	141	32	0.815
48 h fasting + 24 h refeeding	12	266	229	37	0.890
96 h fasting + 24 h refeeding	6	210	130	80	0.619

* Unit: activity per gm (ww) worm.

TABLE VI

In vitro *refeeding studies of the protein contents, glycogen contents, and the activities of glycogen synthase in* H. diminuta *from 48 h starved host*

Host's condition	No. of worms	Protein (% ww)	Glycogen (% ww)	Glycogen synthase activity*			
				Total (T)	I	D	Ratio I/T
No fasting	12	1.6	2.90	942	70	872	0.074
48 h fasting	7	3.9	0.80	2135	245	1890	0.115
48 h fasting + 10 min refeeding	7	4.2	0.36	4288	3034	1254	0.708
48 h fasting + 20 min refeeding	7	3.4	0.20	3916	2869	1046	0.733
48 h fasting + 1 h refeeding	7	2.6	0.18	2375	1771	604	0.746
48 h fasting + 3 h refeeding	7	2.7	1.80	2021	520	1501	0.257

* Unit: Activity per gm (ww) worm.

each group constituting a sample. Each sample was incubated in 20 ml of saline solution containing 1 m*M* glucose equilibrated with 5% CO_2 in nitrogen. The samples were incubated at 37°C in a water-bath shaking at 60 oscillations/min for various periods. Saline was changed once every hour to prevent accumulation of metabolic end products and to replenish glucose.

The results (Table VI & VII) indicated an increase in the glycogen content of the worms incubated in a medium of 1 m*M* glucose. Immediately upon the availability

TABLE VII

In vitro *refeeding studies of the activities of glycogen phosphorylase in* H. diminuta *from 48 h starved host*

Host's condition	No. of worms	Glycogen phosphorylase activity*			
		Total (T)	a	b	Ratio a/T
No fasting	9	237	135	102	0.570
48 h fasting	7	334	231	103	0.692
48 h fasting + 10 min refeeding	7	76	44	32	0.579
48 h fasting + 20 min refeeding	7	81	48	34	0.593
48 h fasting + 1 h refeeding	7	77	39	38	0.506
48 h fasting + 3 h refeeding	7	105	54	51	0.514

* Unit: activity per gm (ww) worm.

of glucose, the total glycogen synthase activity increased while the total phosphorylase activity decreased. There was also an abrupt increase in the I/T ratio of glycogen synthase activities.

DISCUSSION

Fasting the host not only significantly decreases the wet weight and length of *H. diminuta* (Read and Rothman, 1957), it also increases the protein concentration (Goodchild, 1961a) and decreases the glycogen content in the worm (Read and Rothman, 1957; Goodchild, 1961b). The present investigation confirms the above reports (Table I). The apparent increase in protein content in the worm during host fasting may be due to the uneven distribution of non-protein reserve substances in the worm as suggested by Goodchild (1961b). The older reproductive proglottids, which are always the first to be shed during starvation, contain relatively more lipids and polysaccharides (Ip and Fisher, 1982b). This is confirmed by the present observation that the protein contents of the 48-h fasted worms and the unfasted worms artificially reduced to equivalent length are comparable.

When nutrients become limiting during host fasting, the parasite has to metabolize its endogenous glycogen to survive. The glycogen content, therefore, decreases with increasing periods of fasting (Read, 1956; Goodchild, 1961a). However, it leveled off after fasting for 48 h, suggesting that a minimal concentration of glycogen was maintained even under extreme conditions. Since approximately 10% of the worm's glycogen is structural (Beuding and Fisher, 1970), it would be disadvantageous for the worm to utilize all of them. Furthermore, a minimal amount of glycogen is required to serve as primer should food again become available. The marked increase in glycogen content 24 h after refeeding of hosts subjected to various periods of fasting (Table II, IV) suggests that there is a proportionate increase in the efficiency of glycogenesis with starvation. This is in agreement with observations made by earlier investigators (Daughtery, 1956; Read, 1956; Read and Rothman, 1957). The same rapid conversion of glucose to glycogen also is observed in the fasted surrenalectomised rats after feeding with glucose (Cori and Cori, 1927).

In the present study, the total glycogen synthase activities in *H. diminuta* increased during host fasting but the active 'I' form decreased simultaneously. The large amount of inactive glycogen synthase present would act as a reserve which can be rapidly converted to the active form once food is available. Such conversion would be more efficient than *de novo* synthesis of the enzyme. Similar phenomena have been observed in the rat liver (Gruhner and Segal, 1970; Curnow and Nuttall, 1972). The trend, however, reversed itself in worms which had been fasted for more than 48 h; the total synthase activities decreased while the activities of the 'I' form increased (Table II). The total phosphorylase activity as well as the 'a' form, unlike the rat-liver system, increased during host fasting (Table III). Such increase in the activity of 'I' form of synthase and 'a' form of phosphorylase suggests that the synthesis and degradation of glycogen occur simultaneously. As the amount of glycogen present in worms fasted for more than 48 h was very small, the increased activity of the synthase helped to recycle the glucose released by phosphorylase back into glycogen. Such a mechanism prevents the glycogen from being completely used up during prolonged fasting. This explains the leveling off of the glycogen content after 48 h of host starvation. Similar increase in the 'I' form of glycogen synthase has also been reported in the rat (Curnow and Nuttall, 1972; Hue *et al.*, 1975).

Twenty-four hours after refeeding rats which have been fasted for various periods, the activity of glycogen synthase I in *H. diminuta* decreased as compared to those of starved worms. This agrees with observations made on the rat liver (Hornbrook, 1970; Curnow and Nuttall, 1972) but contradicts the results reported by Mied (1975) for the same species of tapeworm. This apparent contradiction may be due to the fact that synthase activities in the present study was measured 24 h after food was made available to the starved rats. Mied (1975) measured the activity immediately after refeeding. *In vitro* refeeding studies however, demonstrated that there was indeed a marked increase in the total synthase activity immediately upon incubation in 1 m*M* glucose indicating that glucose perhaps could be the activator of glycogen synthase in *H. diminuta*. Glucose-induced increase in synthase activity also has been reported for isolated rat livers perfused with glucose, and it appears to be independent of changes in the levels of exogenous hormones (Buschiazzo *et al.,* 1977). Upon further incubation in the presence of glucose, the glycogen synthase activity gradually dropped (Table III). As glycogen accumulates in the worm, the average molecular weight of glycogen increases (Orrell *et al.,* 1966) and the presence of high molecular weight glycogen molecules in the worm inhibits glycogen synthase activity, especially that of the 'I' form (Mied, 1975; Dendinger and Roberts, 1977).

A decrease in both the total and the 'a' form of the phosphorylase activities in *H. diminuta* was observed 24 h after the host was refed. There was also a steep drop in the phosphorylase activity in worms incubated in 1 m*M* glucose after 48 h of starvation. Such decrease in phosphorylase activity suggests that glucose may deactivate the phosphorylase. In mammalian systems this deactivation is effected by the glucose binding to the 'a' form of the enzyme, forming a complex which is a superior substrate for phosphorylase phosphatase. This lowers the phosphorylase activity which in turn slows down glycogenolysis. The removal of phosphorylase 'a', which is a strong inhibitor of glycogen synthase phosphatase, allows the latter enzyme to convert the synthase 'D' into its 'I' form, which determines the ultimate rate of glycogen synthesis (Stalmans *et al.,* 1974; Hers, 1981). Simultaneous deactivation of phosphorylase 'a' and activation of the 'I' form of synthase in *H. diminuta* were observed in the *in vitro* refeeding studies (Tables VI, VII). Whether the deactivation of phosphorylase 'a' is a prerequisite to the activation of glycogen synthase, as proposed by Stalmans *et al.* (1974) for the mammalian system as yet cannot be confirmed.

Glycogen content, glycogen synthase, and phosphorylase activities of artificially reduced worms from unfasted hosts were different from that of normal worms from unfasted hosts indicating differential distribution of the above substances along the length of the worms. When worms from 48 h fasted hosts were compared with worms from unfasted hosts artificially shortened to the length of the former, the glycogen content as well as the enzyme activities were also found to be different (Tables II, III). Such differences cannot be due to the shedding of proglottids alone but is attributable to a biochemical adaptation to starvation.

Acknowledgement

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DISTRIBUTION OF SEROTONIN-LIKE IMMUNOREACTIVITY IN THE CENTRAL NERVOUS SYSTEM OF THE PERIWINKLE, *LITTORINA LITTOREA* (GASTROPODA, PROSOBRANCHIA, MESOGASTROPODA)

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ABSTRACT

The distribution of serotonin-like immunoreactivity was determined in whole-mounted ganglia of the central nervous system of the prosobranch snail, *Littorina littorea,* with the goal of establishing a basis for cellular comparisons between different gastropod nervous systems. While general similarities appear to exist with other species, the nervous system of *Littorina* appears to differ markedly from the nervous systems of the pulmonate and the opisthobranch gastropods with regard to its relatively large number of small, serotonergic cell bodies. Over 1500 cells in *Littorina* were tentatively identified as serotonergic. These cells and their processes were distributed through all the major ganglia, but the vast majority of immunoreactive cell bodies were found in the pedal ganglia. Most cells in the central nervous system were identified only as members of clusters, however, a few could be reliably identified as consistent individuals between preparations. Further histological and physiological studies are needed on prosobranch nervous systems before evolutionary relationships can be established at the cellular level with pulmonate or opisthobranch nervous systems.

INTRODUCTION

The gastropod nervous system has developed through complex evolutionary processes which are poorly understood. A major reason for this lack of understanding is that homologies between nerves, ganglionic lobes, and even whole ganglia, which could serve as the basis for such an understanding, are often not obvious because of changes rendered by cephalization, torsion, and detorsion (Bullock and Horridge, 1965). Recently, however, some insights have been gained from the finer resolution afforded by the study of single identified neurons. For example, the examination of cells which appear to be bilateral homologs, but which appear to lie within bilaterally asymmetrical ganglia, have provided new means for identifying parts of ganglia which may have fused through evolution (Hughes and Tauc, 1963; Hughes, 1967; Munoz *et al.*, 1983). Single cells which are putative homologs have also been identified between species. While the serotonergic metacerebral giant cell is the best studied example of such a neuron in gastropods (Senseman and Gelperin, 1974; Weiss and Kupfermann, 1976; McCrohan and Benjamin, 1980; Granzow and Rowell, 1981; Pentreath *et al.*, 1982; Longley and Longley, 1984; Croll, 1985), evidence suggesting other homologies has also appeared (Dorsett, 1974; Chase and Goodman, 1977; Dickinson, 1980a, b). Such findings may provide useful clues into certain phylogenetic relationships, but

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this body of literature is presently too sparce to serve as a solid basis for understanding broad evolutionary trends in the gastropod nervous system.

One direction in which to expand the present scope of the literature is toward a wider phyletic approach. Over the last two decades, there has been a tremendous expansion in our knowledge of the anatomy and physiology of the gastropod nervous system. To date, however, studies have been relatively restricted within the gastropods, with most examples having been drawn from only a small portion of the existing opisthobranch and the pulmonate genera. Large gaps, therefore, still exist in our knowledge. In fact, very little has changed in intervening years to invalidate the statement of Bullock and Horridge (1965) that only a few "sketchy results are about the only histological studies in the brain in the whole range of prosobranchs" (p. 1359).

As a first effort at building a broader comparative base, the present report demonstrates the location of cell bodies and their processes with serotonin-like immunoreactivity in the central nervous system of the periwinkle, *Littorina littorea.* Since the distribution of serotonergic cells is now established in a number of other gastropods, the purpose of this study is to establish a basis for comparison at the cellular level. Such a comparison has an added evolutionary importance since *Littorina,* a fairly typical mesogastropod prosobranch, is thought to represent the more ancestral form from which both the pulmonates and the opisthobranchs may be derived (Bullock and Horridge, 1965).

Since the periwinkle has not been used as a modern neurobiological preparation, aspects of its gross neuroanatomy are re-examined here and are compared with previous reports as a necessary background for the interpretation of the observed serotonin-like immunoreactivity patterns.

A preliminary report of certain aspects of this study has appeared elsewhere (Croll, 1985).

MATERIALS AND METHODS

Adult *Littorina littorea* were collected locally and were maintained at ambient temperatures (3°C–13°C) in fresh, running seawater supplied by the Dalhousie Aquatron System.

Gross neuroanatomy

Five animals were sacrificed for an examination of the gross anatomy of the central nervous system. After dissection, the ganglia were placed on microscope slides according to their *in situ* orientation, with the exception of the pedal ganglia which were rotated 90° so that the posterior surface faced upward for flat mounting. The ganglia were then stained with methylene blue, dehydrated, cleared and mounted with Permount in preparation for camera lucida tracing.

Immunohistochemical procedures

These procedures were based largely on the indirect immunofluorescent techniques developed by Coons (1958) and subsequently applied to the detection of serotonin-like immunoreactivity by Steinbusch *et al.* (1978). Further refinements for use with whole-mounted invertebrate ganglia were added by Beltz and Kravitz (1983). In addition, the ganglia were treated with protease prior to fixation (see below) to allow for better penetration of antibodies.

For normal immunocytochemical staining, the central ganglia were dissected from approximately 50 snails. The ganglia were cleaned of loose, surrounding tissue but no attempt was made to mechanically remove the inner connective tissue sheath. The ganglia were next immersed in 0.5% protease (Type IV, Sigma Chemical) in phosphate buffered saline (PBS:13.4 g $Na_2HPO_4 \cdot 7H_2O$ and 8.0 g NaCl diluted in distilled water to a final volume of one liter and then adjusted to pH 7.2) for 5–7 min at room temperature. This step was essential for the successful staining of the ganglionic cells bodies although immunoreactivity was seen in fibers in the nerve trunks without proteolosis. The tissues then were transferred from the protease solution directly into 4% paraformaldehyde in PBS at 4°C for 12 h. Following fixation, the ganglia were washed in four one hour baths of antisera diluent (ASD: 0.5% Triton X-100 and 1.0% normal goat serum in PBS) and then incubated for 72–84 h at 4°C in a 1:500 or 1:1000 dilution of primary antibody (rabbit anti-serotonin antibody from Immuno Nuclear Corp., Stillwater, Wisconsin) in ASD with 1.0% bovine serum albumin. No consistent difference in staining was noted between the different dilutions. The ganglia next were washed in four one-hour baths of ASD and then incubated for 24 h at 4°C in a 1:50 dilution of secondary antibody (goat anti-rabbit antibody conjugated to fluorescein isothiocyanate [FITC] from Antibodies, Inc. of Davis, California) in ASD. The ganglia were washed for 12 h in several changes of PBS and then cleared and mounted in 3:1 solution of glycerol in PBS.

The ganglia were viewed and photographed through a Leitz Orthoplan microscope equipped for epifluorescence (H2 filter block with an additional 455 nm long-pass excitor filter). Photographs were made using Kodak Tri-X or Ektachrome 400 (daylight) film.

Controls for antibody selectivity

In addition to the ganglia from animals described above, several different groups of control ganglia were also processed. Except for the changes specifically mentioned below all other procedures for these control specimens were identical to those employed with the normal immunohistochemical preparations. In two groups, the primary anti-serotonin antibody was pre-incubated for 24 h at 4°C with 1 mg of serotonin creatinine sulfate or dopamine hydrochloride (Sigma) per ml of diluted antibody (1:500) prior to incubation of the ganglia. The final concentrations were thus 2.5×10^{-3} M serotonin and 5.2×10^{-3} M dopamine. Of these control ganglia the only ones to subsequently exhibit immunoreactivity were those ones of the group in which the primary antibody was pre-incubated with dopamine, and in this group the pattern of reactivity was similar to that observed following the normal procedure. No attempt was made to determine the minimum concentration of serotonin effective at blocking the staining, however, in a third control group all subsequent labelling was blocked when 1 ml of diluted primary antibody (1:1000) was pre-incubated with 400 μg serotonin conjugated to bovine serum albumin (Immuno Nuclear Corp.). In a fourth control group, incubation of the ganglia with the anti-serotonin antibody was replaced by an equal period of incubation with ASD alone. No fluorescent cell bodies or fibers were observed subsequently although a faint and diffuse fluorescent background was detected. A final control group was processed with the omission of the secondary antibody incubation. No significant fluorescence was subsequently observed in any of these ganglia either.

RESULTS

Gross anatomy of the nervous system

The present findings on the anatomy of the nervous system of *Littorina* are in general agreement with previously published reports (Fretter and Graham, 1962; Bul-

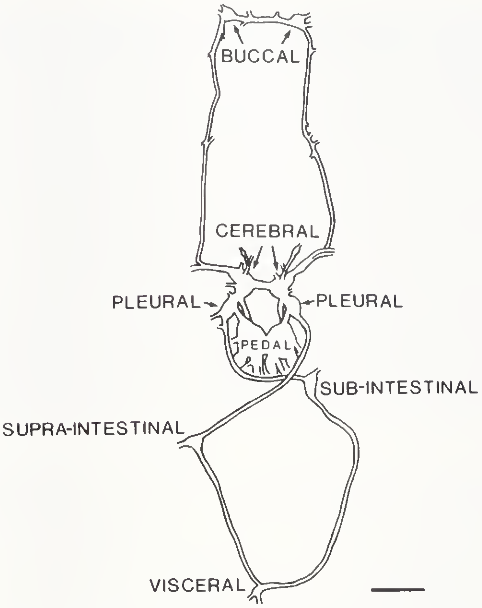


FIGURE 1. Dorsal view of the central nervous system of *Littorina*. Tracing was made from an enlargement of dissected tissue with the ganglia arranged to show approximate relative positions in the whole animal. The pedal ganglia, however, were rotated 90° so that the posterior side is facing upward. All ganglia in subsequent figures are shown in approximately the same orientation unless otherwise stated. Scale bar equals 1.5 mm.

lock and Horridge, 1965). In brief, the paired cerebral ganglia are located *in vivo* just posterior to the buccal mass and superior to the esophagous. These ganglia are connected to the paired buccal ganglia by two long cerebro-buccal connectives (CBC) which course through the superficial muscles of the buccal mass. Several small side branches from the CBC's innervate the buccal mass. Directly posterior to the cerebral ganglia are the two pleural ganglia which are connected to the cerebral ganglia by relatively short connectives. Unlike the other paired ganglia, the pleural ganglia are not interconnected by a commissure. Inferior and slightly posterior to the pleural ganglia are the pedal ganglia. The pedal ganglia are oriented with major anterior and posterior surfaces and with numerous roots exiting ventrally. They are connected to the cerebral and the pleural ganglia by the cerebro-pedal connectives (CPdC) and the pleuro-pedal connective (PlPdC), respectively. Apart from these paired ganglia, three smaller single ganglia are located more posteriorly. The subintestinal ganglion, which is found embedded in the muscle tissue of the right side of the foot, is connected to the left pleural ganglion by the pleuro-subintestinal connective (PlSbC). This connective crosses beneath the pleuro-supraintestinal connective (PlSpC) which connects the right pleural ganglion to the supraintestinal ganglion. The latter ganglion is located in the tissue underlying the left dorsal body wall. This resultant chiastoneury is characteristic of the mesogastropoda (Bullock and Horridge, 1965). The supraintestinal and the subintestinal ganglia are connected posteriorly to the visceral ganglion by the supraintestino-visceral connective (SpVC) and the subintestino-visceral connective (SbVC), respectively. A tracing of the entire central nervous system of *Littorina* is provided in Figure 1.

Localization of serotonin-like immunoreactivity

The distribution of serotonin-like immunoreactivity generally was similar, but not necessarily identical, in all animals. Many individual cells and clusters of cells could be identified reliably between specimens and these features are emphasized in the following descriptions. On the other hand, certain other cells and clusters of cells were noted only in some specimens. These variations are also noted. Using these techniques, a total of approximately 1500 cell bodies have been demonstrated to have serotonin-like immunoreactivity in the central nervous system of *Littorina.* While some neurites often could be traced from cell bodies to their terminations or to the nerve trunks by which they exited the ganglia, in most instances it was difficult to follow single neurites for long distances because they generally ran through neuropilar regions with numerous other immunoreactive elements. Furthermore, observations also were difficult in the cerebral ganglia due to the darkly pigmented cells in the overlying connective tissue.

Cerebral ganglia

Several clusters of cells were identified in the cerebral ganglia (Fig. 2a–e). One group of about 40 cells (the AM cluster) is located on the anterior medial margin of each ganglion (Fig. 2a–d). Cells of the AM cluster appeared to have processes which extend, in a discrete fascicle within the anterior portion of the cerebral commissure, to the contralateral ganglion. In addition to the commissural contributions, fibers also appeared to project from the AM cluster laterally along a prominent S-shaped tract toward the cerebro-buccal connective (CBC; Fig. 2a and 2d). It was not possible, however, to trace the neurite of any single AM cell directly into the CBC. In most instances, it was difficult to discern the outlines of many of the cell bodies in the AM cluster and usually little heterogeneity was seen. However, in certain specimens, two distinct sub-populations were apparent (Fig. 2b). One cluster of 3–5 larger cells lies more posteriorly, near the cerebral commissure, while another group of smaller, more anterior cells comprise the rest of the AM cluster.

Slightly posterior to the AM cluster, another group (the ventral medial or VM cluster) of about 30–40 cells was identified on the ventral surface of each cerebral ganglion. The somata of these cells are smaller than those of the AM group. The VM cluster appeared to have commissural fibers which form a bundle posterior to the afore-mentioned AM commissural fiberes. Some other fibers were traced to a neuropilar region at the lateral margin of the ganglion near a third cluster, the dorsal lateral (DL) group of cells. This DL cluster (Fig. 2b) has approximately 10–15 smaller cells which appeared to have neurites which project laterally toward the cerebro-pedal connective (CPdC).

Apart from these clusters, several prominent single cells also were identified. Among these are single, brightly labelled cells which easily can be identified along the posterior medial margin of each of the hemiganglia (Figs. 2a–c, e). While this pair of cells appeared to be quite consistent in size and shape between animals, exceptions were noted in which either the somatic shape or axonal projections differed. For instance, in Figure 2e, the initial segment of the neurite of one of the cells projects anteromedially rather than anterolaterally as is the case in cells shown in Figures 2a–c. Regardless of these differences, the initial neurite segment consistently branched at a point approximately 140 μm from the cell body. The medial branch then enters the cerebral commissure while the other branch enters the ipsilateral pleural ganglion via the CPdC (also see Fig. 5a). Lateral to these cells, two to four other large cells consis-

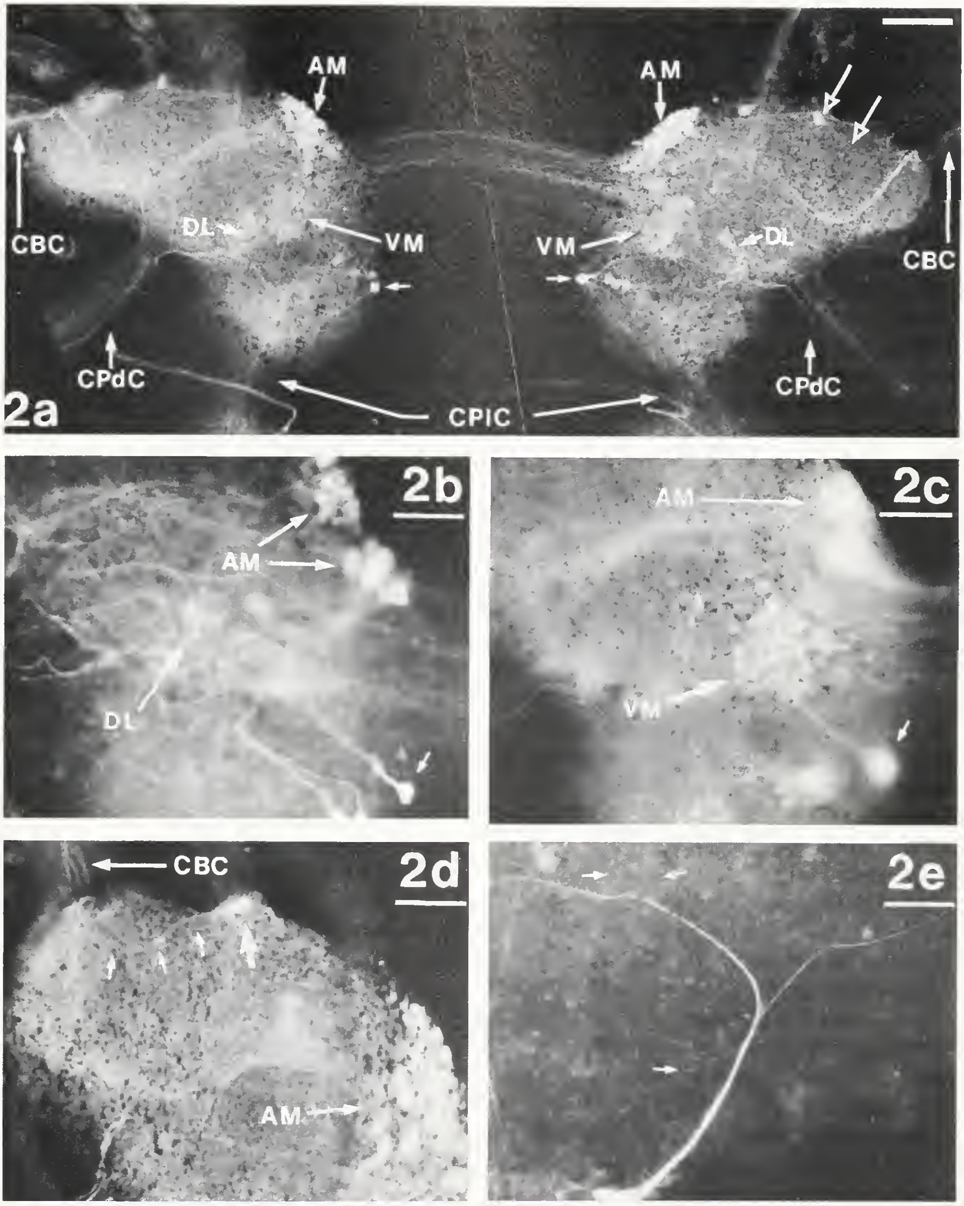
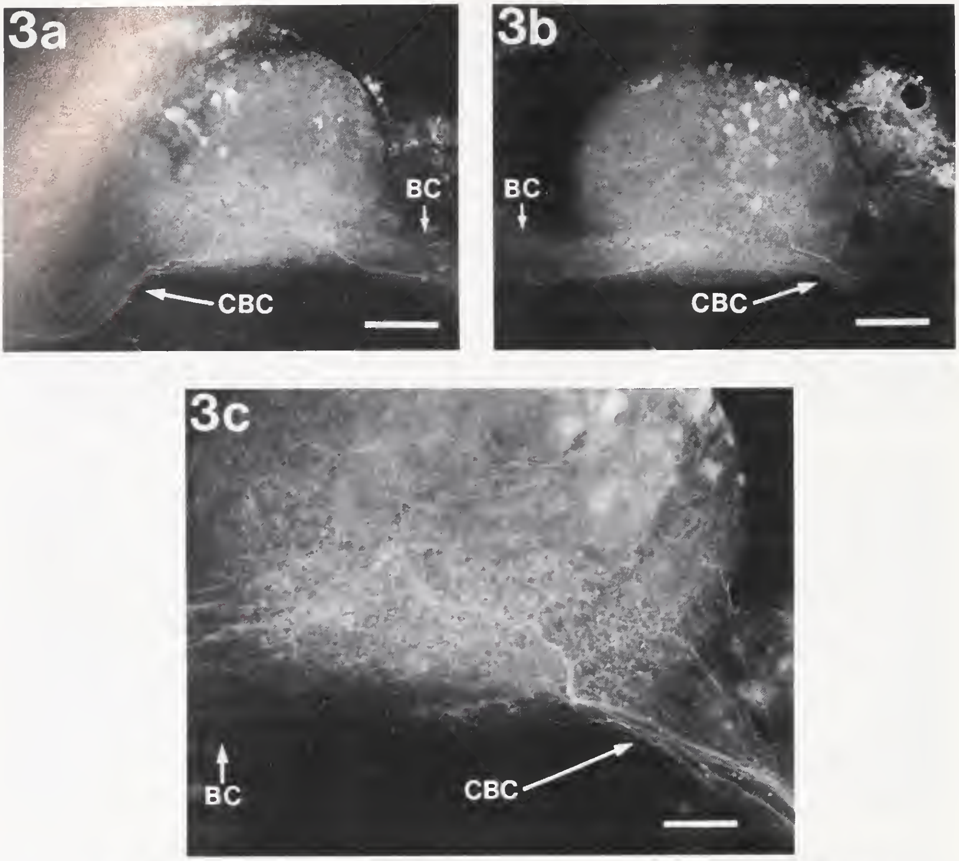


FIGURE 2. Serotonin immunoreactivity of cells in the cerebral ganglia. 2a: Several single cells and cell clusters can be differentiated. These include the anterior medial (*AM*), the ventral medial (*VM*), and the dorsal lateral (*DL*), clusters. Two particularly prominant, bilaterally symmetrical single cells along the posterior medial margin (small closed arrows) and another pair along the anterior margin (larger open arrows pointing to cell body and axon on right side) also can be seen. Interganglionic connectives labelled as follows: *CBC*, cerebrobuccal connective; *CPdC*, cerebropedal connective; *CPlC*, cerebropleural connective. Calibration bar equals approximately 130 μm. 2b–d: Higher magnifications of left cerebral ganglia from different individuals. Orientation is the same as in 2a. 2b and 2c show the DL and VM clusters in relation to the AM cluster and the prominant posterior medial cell (unlabelled arrows). 2d shows the cell body (large arrow) of the single anterior cell with a neurite (small arrows) which projects toward the CBC. Calibration bars for 2b–d = 75 μm. 2e: Details of the branching pattern of the posterior medial cell. Calibration bar for 2e = 45 μm.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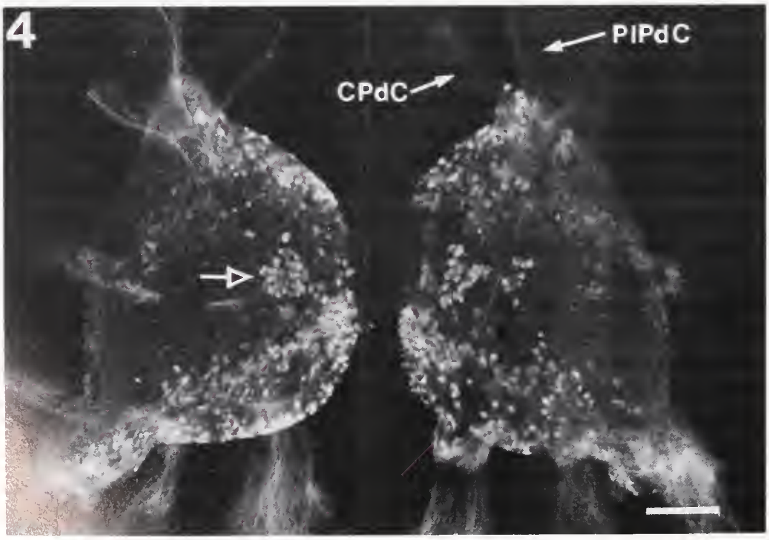
4
PlPdC
CPdC

FIGURE 3. Immunoreactive cells in the left (3a) and right (3b) buccal ganglia. Scale bars equal 100 μm. Higher magnification (3c) shows details of fibers in the buccal ganglion. Scale bar = 44 μm. The buccal commissure, *BC*, and cerebrobuccal connective, *CBC*, are indicated.

tently were located in each cerebral ganglion. The processes of these cells appeared to extend toward the DL cluster as well as the posterior neuropilar region near the CPdC. A final pair of bilaterally symmetrical single cells also were found midway along the anterior margin of the ganglia between the AM cluster and the CBC. The cells were visible in approximately 90% of the specimens. Processes from each of these cells could be traced toward their ipsilateral CBC (Fig. 2a, d), however, despite numerous attempts, it was not possible to trace unmistakably the neurite of this cell directly into the CBC.

Buccal ganglia

Several immunoreactive fibers can be discerned reliably along the lengths of each CBC (Figs. 2a, d and 3a–c). Once inside the buccal ganglia, these processes appeared to have some branches which terminate in the neuropile toward the posterior regions of the ganglia and also collaterals which transverse the buccal commissure toward the contralateral buccal ganglion (Figs. 3a–c).

A total of approximately 20–30 often faintly labelled cells were found in each of the paired buccal ganglia in approximately 75% of the specimens. In the remaining specimens, no cell bodies were discerned although numerous fibers stained prominently. When stained, the cell bodies were located mainly in the anterolateral quadrant of each of the ganglia (Figs. 3a–c). Processes from these cells appeared to project to the neuropile in the posterior part of the ganglia and possibly toward the buccal commissure (BC). Numerous immunoreactive fibers were seen reliably (not shown here) in many of the buccal roots, but it was not possible to determine their origins.

Pedal ganglia

The pedal ganglia have the largest number of immunoreactive cells of all the ganglia. A total of approximately 1000 cells were identified. The cell bodies are rather uniformly small with an average diameter of approximately 8 μm. While the cells lie in a fairly scattered distribution, some bilaterally symmetrical patterns of serotonin-like immunoreactivity could be discerned. One population of cells is distributed over the dorsal third of each pedal ganglion. It extends along the dorsomedial margin from the pedal commissure to the PlPdC. The band of cells then extends laterally along the anterior surface (and therefore is slightly out of focus in Fig. 4) from just dorsal to the pedal commissure to a point midway along the lateral margin of the ganglion. Processes from this population of cells were traced into both the PlPdC and the CPdC as well as a nerve trunk near the dorsolateral corner which innervates the periphery. A second population, forming a discrete cluster of 25–30 cells, is found on the posterior surface of the ganglion just lateral to the pedal commissure. Processes from this cluster were seen projecting principally towards the lateral margins of the ganglia and could be traced toward nerve trunks emerging from those regions. Further ventral, a third popoulation of cells was identified primarily along the posterior surface extending from the medial margin to the dorsolateral corner of the ganglia. This population appeared to be separable into an dorsal and a ventral band by a non-serotonergic region. The ventral band lies along the extreme ventral margin of the ganglion. While

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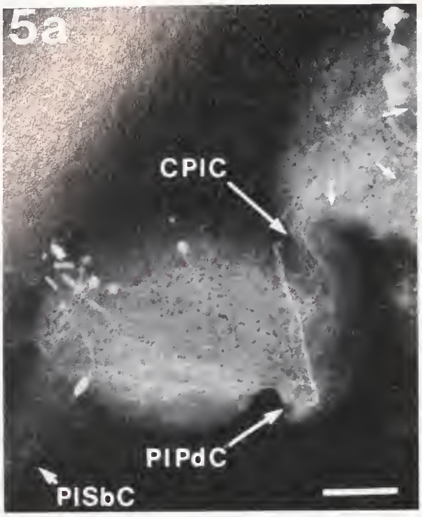
FIGURE 4. Posterior view of the pedal ganglia. Dorsal is to the top of the figure. Open arrow indicates ventral cluster of cells in right pedal ganglion. The cerebropedal connective, *CPdC*, and pleuropedal connective, *PlPdC*, are indicated. Calibration bar = 160 μm.


5a
CPlC
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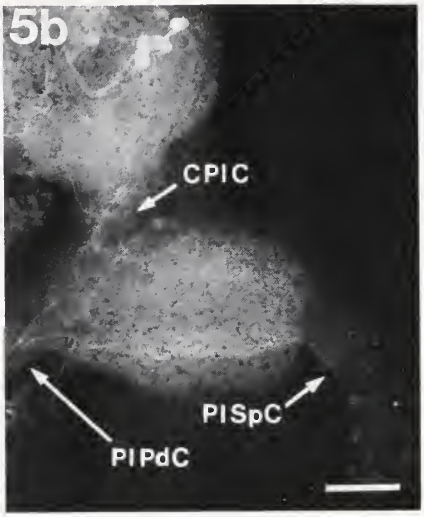
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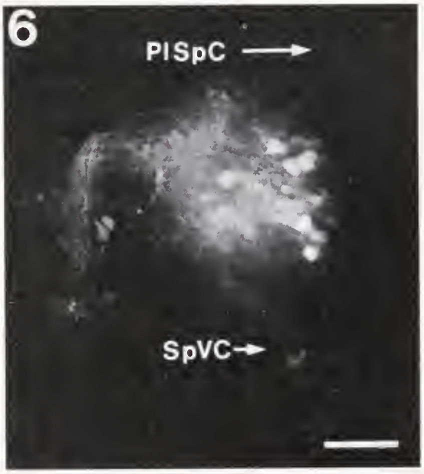
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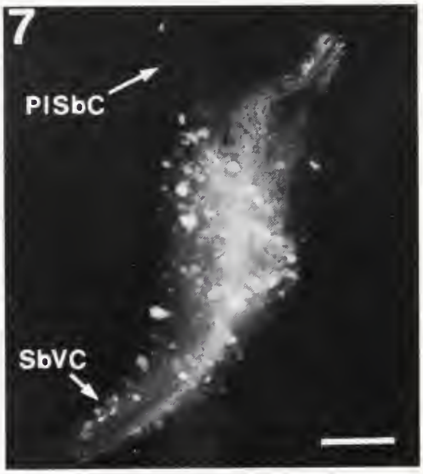
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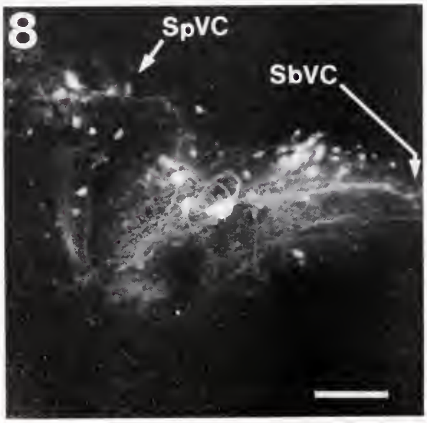
PlSbC
SbVC


8
SpVC
SbVC

a few fibers from the ventral population were found in the ventral region of the pedal commissure, the majority of fibers were found in the nerve trunks emerging from the ventral margins of the ganglia.

Pleural ganglia

Although the two pleural ganglia are similar in size and shape, cell bodies reliably showing serotonin-like immunoreactivity were found principally in the left pleural ganglion. Approximately 10 labelled cells were reliable found in the anterolateral quadrant of this ganglion. Another few cells also were located occasionally along the anterior and the posterolateral margins. Processes from these cells appeared to project widely throughout the neuropile and also toward both the CPlC and the PlPdC (Fig. 5a). Although a few faintly labelled neurons occasionally were detected in the right pleural ganglia of some animals, in the majority of cases, these ganglia were devoid of immunoreactive cell bodies (Fig. 5b). However, labelled fibers reliably were found in all interganglionic connectives and intraganglionic fiber tracts associated with both the left and the right pleural ganglia.

Supraintestinal, subintestinal, and visceral ganglia

A cluster of approximately 40 cells are located along the medial border of the supra-intestinal ganglion (Fig. 6). Processes from this group were traced to the PlSpC and to a nerve trunk which exits from the lateral margin of the ganglion. A few cells were also found scattered lateral to this cluster. Processes from these cells were traced into the SpVC. Another group of 6–8 smaller cells were found near the anterior border of the ganglion. Processes from this group were found mainly in the nerve trunk on the lateral side of the ganglion.

Approximately 80 cells exhibiting serotonin-like immunoreactivity were located in the subintestinal ganglion (Fig. 7). Bundles of fibers were seen in both the PlSbC and the SbVC. Within the ganglion, these fibers appeared to be confined to the neuropile at the center of the ganglion. It was impossible to determine the origins of these fibers.

Numerous immunoreactive cells were found in the visceral ganglion (Fig. 8). In the center of the ganglion, a few cells with relatively large somata were prominent. These cells appeared to have processes extending toward the SpVC, the SbVC, and a nerve trunk exiting the ganglion at the posterior margin of the ganglion. Laterally, a cluster of approximately 30 cells was observed on the right side of the ganglion where the SbVC joins the ganglion. Another population of approximately 10–20 cells was located on the left anterolateral margin where the SpVC joins the visceral gan-

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FIGURE 5. Immunoreactivity in the left (5a) and right (5b) pleural ganglia. Also indicated (small arrows in 5a) is lateral branch of the neurite of prominant posterior medial cell of the cerebral ganglion which projects to the pleural ganglion. Interganglionic connectives are labelled as follows: *CPlC,* cerebropleural connective; *PlPdC,* pleuropedal connective; *PlSbC,* pleurosubintestinal connective; *PlSpC,* pleurosupraintestinal connective. Scale bar = 100 μm.

FIGURE 6. Serotonin immunoreactive cells in the supraintestinal ganglion. The pleurosupraintestinal connective, *PlSpC,* and the supraintestinovisceral connective, *SpVC,* are shown. Scale bar = 100 μm.

FIGURE 7. Immunoreactivity in the subintestinal ganglion. The pleurosubintestinal connective, *PlSbC,* and the subintestinovisceral connective, *SbVC,* are indicated. Calibration bar = 100 μm.

FIGURE 8. Serotonin immunoreactivity in the visceral ganglion. The supraintestinovisceral and the subintestinovisceral connectives, *SpVC* and *SbVC,* respectively, are indicated. Scale bar = 100 μm.

glion. Posteriorly, a few fibers were traced into a nerve trunk which innervates the periphery.

DISCUSSION

Recently developed immunocytochemical techniques provide a simple, reliable method to rapidly compare the nervous systems of diverse species at the level of the single identified cells or clusters of small numbers of cells. Relatively small numbers of cells with distinct biochemical phenotypes can be selectively revealed. When applied to whole-mounted ganglia, these techniques allow for easy visualization of the three dimensional structure of cells in preparations with a close correspondence to the preparations used in physiological studies. Thus they reveal morphology and biochemistry and allow for a correlation with function. The identification of such biochemically discrete single cells or small populations of cells with known functions carries added importance in that it allows for comparisons of specific, analogous, and possibly homologous, cells across phylogeny.

The use of this approach to tentatively identify the serotonergic cells of the gastropod nervous system appears to be particularly promising. Serotonin has long been thought to play an important role in the gastropod nervous system (Gerschenfeld, 1973). More recently, several serotonergic cells or clusters of cells have been identified in diverse species and it has been possible to ascribe a role to many of these cells. For example, activity in the serotonergic metacerebral giant cells modulates the patterned motor output which underlies feeding in numerous gastropod species including *Aplysia* (Weiss *et al.,* 1978, 1979), *Pleurobranchaea* (Gillette and Davis, 1977; Croll *et al.,* 1985), Limax (Gelperin, 1981), and *Helisoma* (Granzow and Kater, 1979). Identified serotonin-containing cells in the pedal ganglia of *Tritonia* initiate locomotion by activating ciliary cells on the sole of the foot (Audesirk, 1978; Audesirk *et al.,* 1979). Evidence suggests that other serotonergic cells function as cardio-excitors (Liebeswar *et al.,* 1975) and others may play a role in re-setting circadian rhythms (Corrent *et al.,* 1978).

Immunohistochemical techniques allow for the initial identification of serotonergic cells and thus serve as a basis for direct comparison of the nervous systems of phyletically distant species such as *Littorina.* However, observations of serotonin-like immunoreactivity must be interpreted in the light of demonstrated selectivity of the procedure. Several lines of evidence indicate that the immunocytochemical techniques employed in this study appear to reliably detect serotonergic cells. The controls within this study demonstrate that selective binding of the primary antibody to tissue antigens underlies this procedure and that this binding of the primary antibody, at the dilutions employed here, can be blocked by pre-incubation with serotonin conjugated to bovine serum albumin or with free serotonin but not with dopamine. This finding is consistent with results found in other invertebrate studies (Beltz and Kravitz, 1983; Goldstein *et al.,* 1984; Klemm *et al.,* 1984; Ono and McCaman, 1984). More direct evidence for the specificity of the immunohistological technique for the demonstration of serotonin in invertebrate nervous systems comes from the close correlation found between the distribution of serotonin-like immunoreactivity in *Aplysia* and the serotonin contents of single identified cells as determined by a radioenzymatic assay (Ono and McCamman, 1984). There have also been good correlations between cells which show serotonin-like immunoreactivity and serotonin-like (yellow) fluorescence obtained through glyoxylic acid and aldehyde demonstrations of biogenic amines in *Aplysia* (Tritt *et al.,* 1983; Ono and McCaman, 1984) and in the cockroach (Klemm, 1983; Klemm *et al.,* 1984). In both animals, however, immunohistochemistry labelled more cells, thereby indicating either its greater sensitivity or lesser selectivity. The former possibility seems more likely given the other available evidence.

Assuming, then, that serotonin-like immunoreactivity is a good indicator of serotonin content, one can make meaningful comparisons in staining patterns between species. A notable feature of the central ganglia in *Littorina* is that many more cells appear to be serotonergic than in representative opisthobranch and pulmonate species. For example, Ono and McCaman (1984) estimated a total of only approximately 120 serotonergic central neurons in *Aplysia* based on their immunohistological studies. Audesirk (1985) appears to have located approximately 200 serotonergic cells in *Lymnaea* based on glyoxylic acid histofluorescence. By contrast, our evidence suggests that the central ganglia of *Littorina* contain a total of approximately 1500 serotonergic cells. While a portion of this total number is composed of cells which exhibited variable labelling intensity and therefore may contain questionable levels of serotonin (see below), the vast majority (over 90%) reliably exhibited intense and unambiguous staining. Therefore it appears that the nervous system of this prosobranch contains 7–10 times as many serotonergic cells as do the nervous systems of representative pulmonates and opisthobranchs.

Another general feature of the nervous system of *Littorina,* which is a well known feature of prosobranchs (Bullock and Horridge, 1965) and which was confirmed here is that the neuronal cell bodies are relatively small as compared to the giant cell bodies found in the nervous systems of the pulmonates and opisthobranchs. While much of the difference in cell size may be accounted for by the small body size of *Littorina,* a comparison of the relative sizes of the serotonergic cell bodies to ganglion dimensions in adult *Aplysia* (Ono and McCaman, 1984), juvenile *Aplysia* (Goldstein *et al.,* 1984), and adult *Lymnaea* (Audesirk, 1985) all emphasize the predominance of relatively smaller serotonergic cells in the nervous system of *Littorina.*

The larger number and the smaller relative sizes of the serotonergic cells indicate that cell-to-cell correspondences may not always exist, or may be difficult to recognize between *Littorina* and any pulmonate or opisthobranch species. However, more general comparisons can be made.

Several clusters of cells and a few identified single cells have been located in the cerebral ganglia of *Littorina.* As in the other gastropod species thus far examined, the cerebral ganglia appear to be bilaterally symmetrical in their serotonergic innervation. Projections from the cerebral ganglia are generally poorly described in other species, however, one well-known serotonergic pathway from the cerebral ganglia involves the metacerebral giant cell (MCG). In other species, the MCG appears to be the only source of serotonin in the buccal ganglion (Ono and McCaman, 1984; Goldstein *et al.,* 1984, Murphy *et al.,* 1985). Fibers from the cerebral ganglia appear to be a major but perhaps not the only source of serotonergic innervation to the buccal ganglia in *Littorina.* However, unlike the case in other species where the only serotonergic fiber in the each of the CBC's is the single, large axon of the MCG, in *Littorina* each of the CBC's contains several fibers with serotonin-like immunoreactivity. The source(s) of these fibers is still unclear but they may arise from one or more of the following three sources: (1) a single, isolated cell body lying midway along the anterolateral margin of the cerebral ganglion, (2) cells within the AM cluster of the cerebral ganglion, or (3) a population of cells residing within the buccal ganglia. With regard to this last cell population exhibiting serotonin-like immunoreactivity, the buccal ganglia of *Littorina* appear to differ from the buccal ganglia of the other species thus far examined, where no evidence exists for serotonergic cell bodies in normal animals. Interestingly, however, Audesirk (1985) found that when the buccal ganglia of *Lymnaea* were pre-incubated in 5-hydroxytryptophan before histological processing, a small number of cells subsequently showed serotonin-like fluorescence induced by glyoxylic acid. No serotonergic cell bodies were detected in the buccal ganglia if the pre-incubation was omitted. This evidence suggests that certain cells in the buccal ganglia of *Lymnaea* differ in their uptake of this serotonin precursor. It is unknown whether these cells subsequently synthesized serotonin since glyoxylic acid induced histofluorescence does not allow for discrimination between 5-hydroxytryptophan and serotonin.

While serotonin-like immunoreactivity appears to correspond generally well with serotonin content, it must be noted that cells in the buccal ganglia were variable in their staining intensity. They were often labelled only faintly and often were unlabelled. Despite this variability, the immunoreactive fibers in the buccal roots and neuropile showed relatively little variability. Ono and McCaman (1984) reported a certain degree of variability in both serotonin-like immunoreactivity and in serotonin content of individual neurons throughout the central ganglia of *Aplysia.* It is unknown whether physiological changes in precursor availability may affect the distribution of serotonin-like immunoflourescence in gastropods but these data suggest a possible correspondence between the buccal cells in *Littorina* which show variable serotonin-like immunoreactivity and buccal cells in *Lymnaea* which can express selective serotonin precursor uptake and/or serotonin synthesis capabilities.

In other species the pedal ganglia appear to contain the largest number of serotonergic neurons. Certain of these cells may be involved in ciliary locomotion in both *Tritonia* (Audesirk, 1978; Audesirk *et al.,* 1979) and *Lymnaea* (Audesirk, 1985). There is also some evidence that serotonin may be involved in controlling the locomotory activity emerging from the pedal ganglia of *Aplysia* (MacKey and Carew, 1983). In *Littorina* the pedal ganglia are also the major source of innervation of the foot (Fretter and Graham, 1962; Bullock and Horridge, 1965) and the pedal ganglia contain the largest number of cells with serotonin-like immunoreactivity. No physiological role for serotonin in *Littorina* is known at present.

The pleural ganglia seem to have very few serotonergic cell bodies, in other gastropod species. Relatively few serotonergic cells were found in the pleural ganglia of *Littorina* as well. However, a few cells were reliably labelled. Interestingly, among the paired anterior ganglia, which all superficially appear to be bilaterally symmetrical, only the pleural ganglia are asymmetrical in serotonin-like immunoreactivity distribution. This asymmetry is presumably a reflection of the fact that the more posterior ganglia which show very little bilateral symmetry are connected directly to the pleural ganglia. These posterior ganglia, the supraintestinal, subintestinal, and visceral ganglia, each have numerous elements exhibiting serotonin-like immunoreactivity. Similarly serotonin appears to be widely distributed in the more posterior ganglia innervating the viscera of other gastropods (Goldstein *et al.,* 1984; Ono and McCaman, 1984; Audesirk, 1985). Unfortunately, too little is presently known about the innervation patterns of the viscera in *Littorina* to make any meaningful comparisons with other species.

The present paper demonstrates that immunocytochemical techniques for the localization of serotonin can provide a basis for comparisons of phyletically distant species. The technique reveals general serotonin distribution patterns within central ganglia and in some cases can allow for the identification of individual cells. Details of neurite projections and arborizations also can be visualized. Further information on the physiological function of these neuronal elements are needed. Further studies on other prosobranch nervous systems also are needed in order to test the generality of the findings reported here. Work presently in progress is focused towards this goal.

It is expected that such studies on the localization of discrete neurochemical substances, such as serotonin, and accompanying physiological studies conducted in a broader phyletic survey will yield new information on the evolutionary trends which have shaped the gastropod nervous system.

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GILL DIMENSIONS IN PELAGIC ELASMOBRANCH FISHES

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ABSTRACT

The warm-bodied great white shark, Atlantic shortfin mako, and common thresher shark exhibit larger total gill surface areas than do ectothermic blue sharks, sandbar sharks, dusky sharks, or scalloped hammerhead sharks. The means by which the three former species have achieved this greater gill surface area differ. Total filament length per given body size in the great white shark is greater than in any other species of elasmobranch examined. In contrast, the Atlantic shortfin mako and common thresher sharks appear to rely upon larger secondary lamellae area as a means of increasing total gill surface area. None of the three species exhibit spacing of secondary lamellae which differ significantly from the arrangements found in the ectothermic species of elasmobranchs. Larger gill surface areas per unit body weight allow for greater volumes of water to be used effectively in the transference of oxygen to the blood, thereby increasing the total amounts of oxygen available to support the high energy physiology of the warm-bodied species.

INTRODUCTION

Measurements of gill dimensions for the purpose of estimating total area of respiratory surface have been made for a number of species of teleosts (notably Gray, 1954; Hughes, 1966; Muir, 1969; Muir and Hughes, 1969; Hughes and Morgan, 1973). Corresponding studies on elasmobranchs are fewer, and are confined to small and/or more sedentary species (Boylan and Lockwood, 1962; Hughes and Wright, 1970; Hughes, 1972; Hughes and Morgan, 1973). In addition, very few of the gill studies to date (teleosts or elasmobranchs) have utilized sufficient numbers of individuals within a species to investigate the relationships between surface area and body weight. Lamnid sharks are warm-bodied (Carey and Teal, 1969a; Carey *et al.,* 1982) in a manner similar to tunas (Carey, 1969b; Carey *et al.,* 1971). The latter group possesses unusually large gill surface areas (Muir and Hughes, 1969), approaching respiratory area estimates of mammalian lungs (Tota, 1978). No attempts have been made to estimate gill surface areas for any of the lamnid sharks, nor measurements conducted to provide estimates of gill areas in active, pelagic species of shark which have not developed an endothermic metabolism. The present report gives measurements of gill dimensions and estimates of total gill surface area in two warm-bodied elasmobranchs, the great white shark (*Carcharodon carcharias*) and Atlantic shortfin mako (*Isurus oxyrinchus*); one suspected endotherm (Carey, 1982), the common thresher shark (*Alopias vulpinus*); and four species of active, pelagic, ectothermic sharks: the

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sandbar (*Carcharhinus plumbeus*), dusky (*C. obscurus*), blue (*Prionace glauca*), and scalloped hammerhead shark (*Sphyrna lewini*).

MATERIALS AND METHODS

Gills were removed from freshly killed specimens of known length and weight and preserved in 10 percent buffered formalin. Total gill surface areas were estimated by the methods of Hughes (1966) and Muir and Hughes (1969):

$$\text{Total Surface Area (TSA)} = 2(L \times d)bl$$

Where L is the total length of all filaments (mm), d is the number of secondary lamellae per mm on one side of a filament, and bl is the surface area (mm^2) of both sides of an average secondary lamella. For each gill arch, the total number of filaments was counted, and the total filament length determined using a dial caliper. The surface area of an individual secondary lamella was measured by tracing its image from a Nikon microscope equipped with a camera lucida microprojection head and subsequently tracing that drawing with a Lasico Model N-30 Planimeter.

Secondary lamellae were sampled from every twentieth filament on the first holobranch. Counts of secondary lamellae were made over the length of these filaments with a microscope equipped with an ocular micrometer (minimum of six counts of lamellae falling within the field of the micrometer from the base to the tip of the filament). A minimum of three lamellae from three different levels on the filament were carefully removed for determination of surface area: one from the base of the filament, one from the middle, and one from the tip. Secondary lamellae were removed with a scalpel and/or razor blade.

The first holobranch was examined in every specimen and complete gill sets were examined in: 4 *Alopias vulpinus;* 6 *Carcharodon carcharias;* 4 *Isurus oxyrinchus;* 4 *Carcharhinus plumbeus;* 3 *Carcharhinus obscurus;* 4 *Prionace glauca;* and 2 *Sphyrna lewini*. From these complete sets, the mean percentage of total filament length attributable to the first holobranch was calculated. This value was used to estimate total filament lengths for those animals of the same species from which only the first holobranch had been obtained. The range of percentages never varied more than 1.2 percent for any species.

Additional measurements were made to estimate errors in the following measurements: (a) filament length; (b) counts of secondary lamellae; (c) variations between left and right sides of gill sets; and (d) surface area measurements of secondary lamellae. Errors in measuring filament length using every twentieth filament were never greater than 3%, based on actual measurements of every filament from five randomly selected holobranchs. Errors in counts of secondary lamellae were never significant ($P > 0.05$), based upon counts of secondary lamellae along the lengths of center filaments from all holobranchs within a gill set from eight animals. Variations in filament length, secondary lamellae numbers, and surface areas between left and right gill sets were never more than 1%, based on comparisons of first holobranchs from five animals. Errors in surface area determinations were the largest source of error in this study, but were a function of the difficulties involved in removing entire lamellae, rather than from variations between gill arches. Extra care in secondary lamellae removal minimized this source of error (based on area determinations of secondary lamellae from center filaments of all holobranchs within a gill set from eight animals).

RESULTS

A log/log plot of total gill surface area *versus* body weight is presented in Figure 1 for six of the seven species studied. No regression line is shown for the scalloped

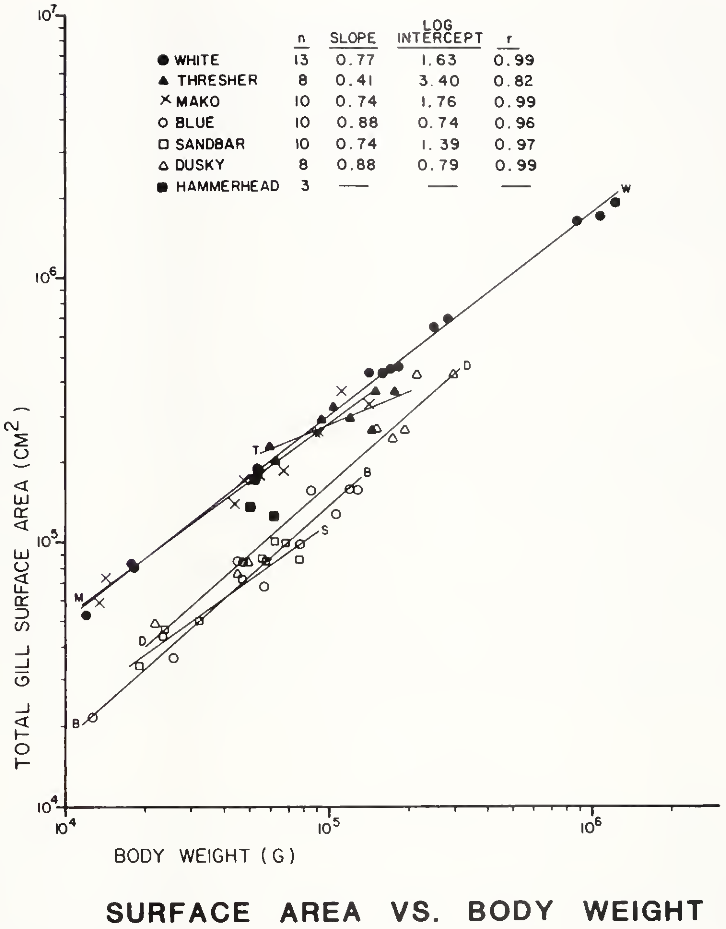

FIGURE 1. Linear regression lines demonstrating the relationships between total gill surface area (cm^2) and body weight (g) in six species of elasmobranchs.

hammerhead in Figures 1 through 4 due to the small number of animals available (three). The regression line for the common thresher, a species suspected to be endothermic (Carey, 1982), exhibits a radically different slope from the remaining five species, and results from the small range in body size of the specimens available for analysis. Differences in regression coefficients were too large to allow for statistical comparisons of all six species together. Only the shortfin mako and sandbar sharks were homogeneous with respect to regression coefficients [SS = 0.102 < SScrit .05 (5, 47)]. The shortfin makos exhibited larger total surface areas in comparison with sandbar sharks ($P < 0.05$).

Total surface areas in all six species were compared using the regression equations presented in Figure 1 to compute the TSA for each species at a common body size (Table I). At a body weight of 100 kg the $\hat{y}$ (estimated TSA based on the regression equation) for the white, mako, and thresher sharks are approximately twice the estimates for TSA obtained for the sandbar, blue, or dusky sharks.

In comparing total filament lengths (Fig. 2), only the great white and mako sharks

TABLE I

Gill dimensions at 100 kg weight (based upon regression equations shown in Figures 1–4)

Species	Total surface area (cm^2)	Total filament length (cm)	Weighted average number sec. lam. per mm	Weighted average sec. lam. area, 2 sides (mm^2)
Greak white shark	301,122	27,096	9.80	5.66
Common thresher shark	281,838	17,644	9.32	8.41
Atlantic shortfin mako shark	229,896	15,983	10.42	8.41
Dusky shark	154,882	17,758	10.89	4.23
Blue shark	138,038	12,367	9.54	5.76
Sandbar shark	123,027	13,051	10.44	4.60

had slopes similar enough to compare statistically [SS = 1.000; <SScrit 0.01 (5, 47)]. The great white shark exhibited significantly larger filament lengths than the mako at $P < 0.05$. From a qualitative view, total filament lengths in the great white shark are larger than those of any other species of shark (Table I).

However, the regression line of the white shark for the number of secondary lamellae per mm (Fig. 3) is not significantly above or below the regression lines of several of the ectothermic species (ANCOVA $P > 0.05$, combined with *a posteriori* STP [white shark, dusky shark SS = 0.921; <SScrit 0.05 (5, 47)] and sum of squares STP tests). At a body weight of 100 kg (Table I) the estimated values for the number of secondary lamellae per mm in the different species are similar ($P > 0.05$).

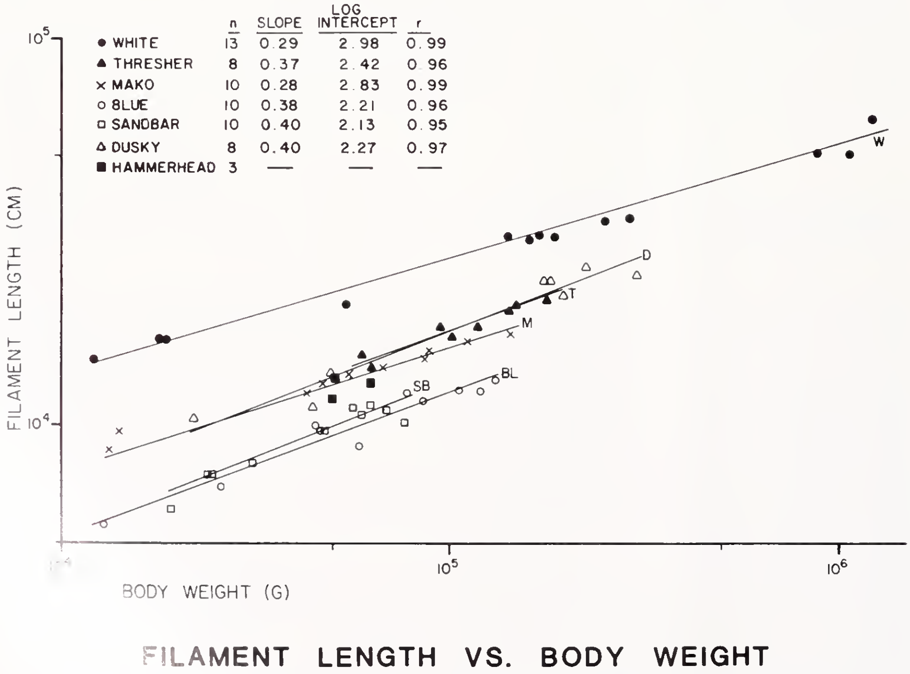

FIGURE 2. Linear regression lines demonstrating the relationships between total gill filament lengths (cm) and body weight (g) in six species of elasmobranchs.

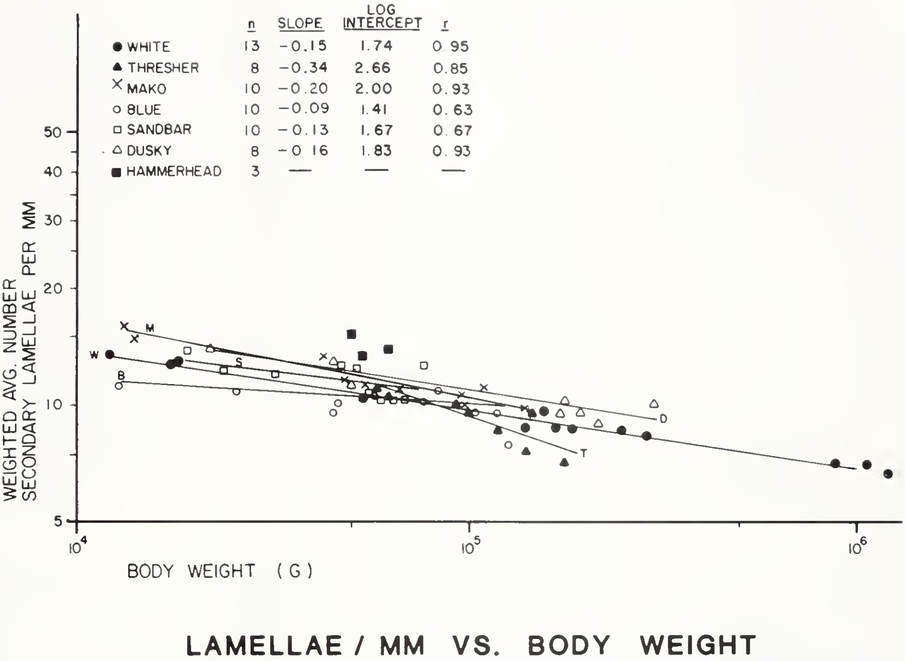

FIGURE 3. Linear regression lines demonstrating the relationships between the weighted average number of secondary lamellae per mm and body weight (g) in six species of elasmobranchs.

The regression line of the weighted average secondary lamellae area *versus* body weight (Fig. 4) for the white shark is not significantly different from the lines of one or more ectothermic species (ANCOVA $P > 0.05$, combined with *a posteriori* STP tests). At 100 kg (Table I) the estimated weighted average surface area of a secondary lamella is 5.66 mm^2 for a white shark, which is virtually indistinguishable from the estimated value of 5.76 mm^2 for a blue shark.

In contrast to the great white shark, our results indicate that the mako relies upon larger secondary lamellae to increase its total gill surface area (Fig. 4). A comparison of the regression coefficients for the mako and dusky sharks [SS = 0.028; <SScrit 0.05 (5, 47)] indicates that the mako possesses significantly larger secondary lamellae. Using $\hat{y}$ at 100 kg body weight (Table I) the shortfin mako has an average lamellae area of 8.41 mm^2, well above the estimated value for the next closest species.

The shortfin mako does not exhibit unusually large filament lengths (Table I and Fig. 2), nor a greater number of secondary lamellae per mm, (Table I, and Fig. 3) according to both statistical (ANCOVAs plus STP procedures) and qualitative methods of comparison.

The small size range and radically different regression coefficients for the thresher shark precludes meaningful statistical comparisons. Qualitative examinations of Table I and Figures 1 through 4 suggests that this species uses a similar method to that of the shortfin mako to increase its total surface area over the values estimated for the non-lamnid species.

The gill structure within the seven elasmobranchs studied is characterized by a high degree of interspecific conservativism. In addition to the similarities in lamellar spacing and size, no radically different secondary lamellar shapes were found between

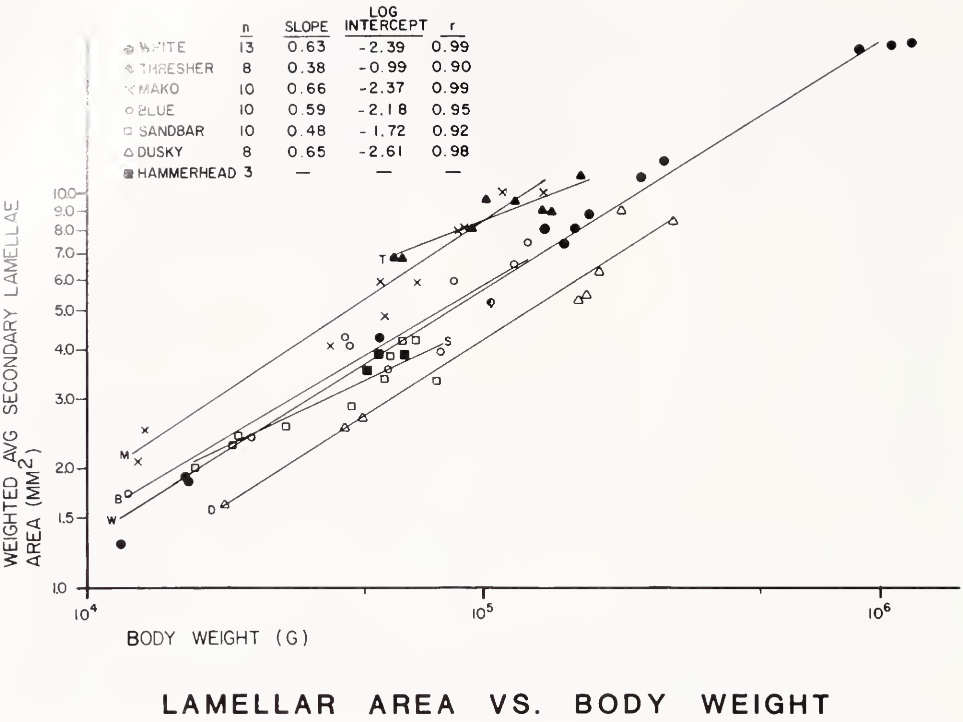

FIGURE 4. Linear regression lines demonstrating the relationships between the weighted average secondary lamellae area (mm^2) and body weight (g) in six species of elasmobranchs.

species at any given sampling location along a filament. Even the percentage of total filament length accounted for by the first holobranch varied less than 2% among all seven species.

No secondary lamellar fusion was noted in any individual gill examined. Filament fusion was occasionally observed in individuals of *all* species. The cause of filament fusion (developmental, parasitic, exposure to pollutants, *etc.*) is not obvious from macroscopic examinations.

DISCUSSION

From both a qualitative and quantitative viewpoint, the warm-bodied and presumed warm-bodied species of shark exhibit larger total gill surface areas than do active, pelagic, ectothermic species of shark. These results parallel those reported by Muir (1969), Muir and Hughes (1969), Randall (1970), and Hughes and Morgan (1973) for teleost fishes, in which warm-bodied tunas were found to possess greater surface areas than those of other species of ectothermic teleosts. Such large surface areas undoubtedly are necessary to facilitate adequate levels of oxygen uptake (Jones and Randall, 1978) to support the functionally endothermic condition (Carey and Teal, 1966; Stevens and Carey, 1981) and associated high metabolic rates (Brill *et al.*, 1978) found in these fish.

There are no studies on large elasmobranchs in the literature to facilitate comparisons to our data on gill surface areas. Hughes and Morgan (1973) list the total surface

TABLE II

Extrapolations of total gill surface areas per gram body weight in selected elasmobranch and teleost species

Species	Surface area per gram at 1 kg (cm^2/g)	Reference
Endotherms		
Skipjack tuna	18.40	Muir and Hughes, 1969
Yellowfin & bluefin tuna	14.38	Muir and Hughes, 1969
Atlantic shortfin mako	9.40	
Great white shark	8.72	
Ecotherms		
Sandbar shark	4.11	
Dogfish	3.70	Hughes and Morgan, 1973
Dusky shark	2.79	
Blue shark	2.39	
Smallmouth bass	1.96	Price, 1931, as presented by Muir and Hughes, 1969
Roach	1.29	Muir and Hughes, 1969

area of a 1 kg dogfish shark (*Squalus acanthias*) at 3.7 cm^2/g. This value, together with weight-specific values for tunas (Muir and Hughes, 1969), smallmouth bass (Price, 1931, using the regression line from Muir and Hughes, 1969), and roach (*Rutilus rutilus*) (Muir and Hughes, 1969) are presented in Table II, and are compared with values based on extrapolations down to 1 kg for all six of the species involved in this study. Values for the two warm-bodied shark species are substantially above those values for the ectothermic teleosts and elasmobranchs but do not equal those values found in tunas. Based upon the comparisons shown in Table II, plus information presented by Gray (1954), gill surface area values for the sandbar, blue, and dusky sharks fall within the range of most teleost fishes.

The way in which the endothermic lamnid sharks and tunas have successfully increased the surface areas of their gills as compared with non warm-bodied species differ. The tunas exhibit significantly larger total filament lengths per unit of body weight combined with increased numbers of secondary lamellae per mm to increase total surface area (Muir and Hughes, 1969). Individual secondary lamellae in tuna are smaller per unit body weight than in more sluggish teleosts (Muir and Hughes, 1969). The warm-bodied sharks do not exhibit increased numbers of secondary lamellae per mm nor smaller secondary lamellae per unit body weight. Only the great white shark exhibits larger total filament length. The mako and thresher do not.

Explanations for these differences between the lamnid sharks and the tunas are not obvious. It is possible that the extended gill septum in the elasmobranchs limits lamellar variability. It is clear that weight specific secondary lamellar numbers do not vary greatly among elasmobranch species. While secondary lamellar areas were significantly greater in the shortfin mako, this is the parameter in which the largest potential variability exists with respect to measurement error. Most teleost fishes have gill filaments free from connection with a septum for most of their lengths. The greater freedom allowed the teleost filaments may enhance the possibility for secondary lamellar enlargement and variation in spacing of lamellae on filaments.

Total gill filament numbers appear to be a species specific characteristic within the sharks we have observed. There are no trends of increases or decreases with body size. The percentage of the total number of filaments within each species accounted for by the first holobranch remained relatively constant. These findings suggest that gill filament number could prove useful in certain species identifications, where other morphological examinations prove inconclusive.

The range of total filament numbers between the species is not large, in contrast to teleosts (Hughes and Morgan, 1973). The lowest estimate of filament count was found in a sandbar shark (1228 per side), the highest in a great white shark (1927 per side). There is no evidence to suggest that the endothermic species possess more filaments per se. Of the species we have examined, the ranking for total gill filament number is as follows (number ± SD per side): great white shark (1892 ± 27); dusky shark (1800 ± 49); common thresher shark (1483 ± 19); shortfin mako shark (1436 ± 32); scalloped hammerhead (1424 ± 25); blue shark (1304 ± 41); and sandbar shark (1249 ± 29). Hughes and Morgan (1973) reported total filament numbers of 749 in *Raja clavata* (0.5 kg) and 1000 in *Squalus acanthias* (1 kg). These data, in addition to our own, suggest that within the elasmobranchs at least, total filament number may be related to the maximum size limits of a species rather than to its ecology or physiology.

It is clear that the functionally endothermic species of shark exhibit larger total gill surface areas than do the non-endothermic forms. Because of the large size differences involved, direct comparisons between the warm-bodied sharks and the tunas are extremely difficult to make. From the limited data available (Table II), it appears that functionally endothermic sharks do not exhibit weight specific TSA values as high as those in tuna. To the extent that these morphological measurements provide insight into physiological performance levels, it may be that the lamnid sharks do not exhibit weight specific metabolic rates as high as those found in tuna and consequently cannot maintain as large a temperature gradient between the swimming musculature and the water as do tuna. The validity of these assumptions needs to be tested using both morphological and physiological information.

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COLD-INDUCED APOLYSIS IN ANECDYSIAL BRACHYURANS

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ABSTRACT

Several integumentary tissues of brachyuran crabs separate from the exoskeleton (undergo apolysis) when exposed to low temperatures (0°C) for one hour or longer. Apolysis did not occur in crabs held at room temperature for the same length of time following the destruction of the subesophageal ganglia *i.e.,* the phenomenon was not due to the death of the animals. Apolysis did occur in animals chilled following ganglionectomy. Cold-induced apolysis occurred in species of seven families of brachyurans distributed throughout tropical, North Atlantic, or Pacific Northwest habitats. Therefore, it is not merely due to chilling subtropical animals. Adult majid crabs, which are in terminal anecdysis, underwent cold-induced apolysis as did Bermuda land crabs, the latter in all stages of the molt cycle tested. Chilling *in vitro* also induced apolysis in isolated pieces of integumentary tissue. The biological significance of these observations is that the mechanisms underlying apolysis may be activated in the integumentary tissues of anecdysial animals.

INTRODUCTION

One of the most definitive events during the crustacean molt cycle is the separation of the epithelium from the membranous layer (ML), the innermost layer of the exoskeleton (Skinner, 1962, 1985); this process has been termed apolysis (Jenkin and Hinton, 1966). Apolysis is one of the earliest responses to increases in titers of the molting hormone, 20-OH ecdysone, that occur in proecdysis (see Skinner, 1985, for references). In the normal molt cycle, apolysis signals the initiation of some of the major metabolic and physiological processes that occur prior to ecdysis. In large specimens of Bermuda land crabs, *Gecarcinus lateralis,* apolysis occurs approximately one month before ecdysis following which the ML and ~75% of the endocuticle are degraded and the epicuticular and exocuticular layers of the new exoskeleton are formed (Skinner, 1962).

We describe here a phenomenon we call cold-induced apolysis. Specifically, we have observed that the epithelium and underlying muscle from chelae as well as the integumentary tissues of branchiostegite regions can be detached easily and cleanly from the exoskeleton if animals are chilled on ice for 0.5 to 4.5 h. The phenomenon also occurs in autotomized chelae and excised pieces of branchiostegites similarly treated. Cold-induced apolysis was observed in members of seven families of brachyurans, including tropical land crabs as well as a number of species of marine crabs that commonly experience temperatures of 10°C and less; it was not observed in

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specimens from the two astacuran families that were examined. These observations imply that the mechanisms underlying at least some aspects of apolysis are present in the integumentary tissues of anecdysial animals. Only a trigger is needed for their release.

Cold-induced apolysis facilitates the recovery of intact crustacean soft tissues and provides a useful method in the analysis of such tissues. Brief reports of some of these results have been published (O'Brien *et al.,* 1984; O'Brien and Skinner, 1985).

MATERIALS AND METHODS

Animals

Specimens of *G. lateralis* were from Bermuda and *Rhithropanopeus harrisii* specimens were from the Chesapeake Bay, Maryland, or near the Duke Marine Laboratory, Beaufort, North Carolina. *Pugettia producta, Taliepus nuttallii, Cancer antennarius,* and *Pachygrapsus crassipes* were collected from Santa Barbara, California. *Gecarcinus ruricola, Cardisoma guanhumi,* and *Menippe mercenaria* were taken from Fort Pierce, Florida. *Callinectes sapidus, Ocypode quadrata, Uca pugilator,* and *Sesarma cinereum* were collected near the Duke Marine Laboratory. Crayfish [*Cambarus bartonii bartonii* (Cambaridae)] were collected locally and lobsters [*Homarus americanus* (Nephropidae)] were purchased from a local market.

Treatment of animals

Assays on *G. lateralis* and *R. harrisii* were performed in Oak Ridge where these two species were maintained. All other assays were performed near the collecting sites.

Experimental animals were packed in shaved ice. The subesophageal ganglia of some control animals were destroyed with a dissecting needle and the animals were placed in a humidified environment at 23°C or chilled in ice until they were dissected. We scored as positive a clean and complete separation of (i) the carapace from the muscles of the cardiac stomach when the carapace was lifted from the posterior border or (ii) the exoskeleton from the underlying integumentary tissues or muscle.

Histology and electron microscopy

Tissues were removed from stage-C_4 *G. lateralis* for examination by light and electron miscroscopy. In the *in vivo* treatments, whole animals were chilled before branchiostegite segments were removed for fixation. Segments of branchiostegites including calcified exoskeleton, ML, and integument were taken with a small rotary power saw. Segments of branchiostegites were removed from five animals for studying *in vitro* cold-induced apolysis; they were placed in sealed plastic bags or plastic wrap before being packed in ice. Following treatment, the ML was peeled from the calcified exoskeleton before fixation. For light microscopy, samples were fixed in Bouin's solution, cut to a thickness of 5 μm at 50 μm intervals, and stained with hematoxylin-eosin. For electron microscopy, samples were fixed 2 h in 2.8% ultrapure glutaraldehyde, 0.57 *M* glucose, and 0.10 *M* sodium cacodylate, pH 7.4, and postfixed 2 h in 2% osmium tetroxide, 0.57 *M* sucrose, and 0.10 *M* sodium cacodylate, pH 7.4 (Mykles and Skinner, 1981). Sections were stained with uranyl acetate and lead citrate and examined with a Philips 400T electron microscope operated at 80 kV.

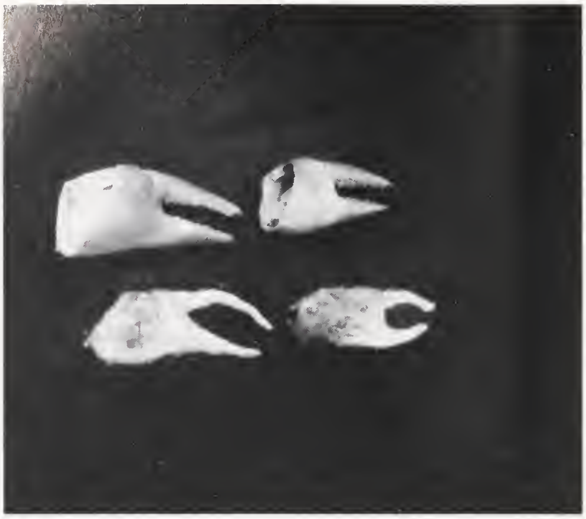

FIGURE 1. Muscles (bottom) removed from propoda and dactyls of autotomized chelae of two *G. lateralis* following 4 h at 0°C. Muscles have also separated from the apodemes which are visible inside the exoskeletal "gloves" (top).

RESULTS

Cold-induced apolysis of the epithelium of the chelae in *G. lateralis* required 3 to 4 h (Fig. 1). Separation of the integumentary tissues occurred in both intact whole animals (Fig. 2A; Table I) and those dying following ganglionectomy when chilled at 0°C (data not shown). Integumentary tissues also separated from the ML when they had been removed from crabs and chilled *in vitro* (Fig. 2B). Large segments of epithelium and groups of tendinal cells (Koulish, 1973) appeared intact after separation. The amount of epithelial tissue adhering to the ML varied considerably even within one individual and following treatment either *in vivo* or *in vitro*. There was little or no cellular debris attached to some areas of the ML (Fig. 3A) while other areas had epithelial cells and parts of tendinal cells present (Fig. 3B, C). Cellular debris remained with ~50% of the microscopic fields of the ML of *in vitro* preparations of branchiostegite regions. In unchilled branchiostegite segments taken from specimens of *G. lateralis* before exposure to 0°C (Fig. 4A) and from dying (ganglionectomized) animals and kept at room temperature (Fig. 4B), the epithelial and tendinal cells of the integument remained attached to the ML. In summary, separation always occurred in chilled tissue and only in chilled tissue.

In electron micrographs of segments of ML from animals induced to undergo apolysis *in vivo*, integumentary tissues had separated completely. Cells appeared to be intact and there was no evidence of lysis. A flocculent material similar to that found in hemolymph sinuses (Fig. 5A) was present in the space between the ML and integumentary tissue; it may have been hemocyanin that had precipitated during fixation. The epithelium of untreated animals (Fig. 5B) as well as of dying ganglionectomized animals kept at room temperature was firmly attached to the ML. Tendinal cells were filled with microtubules whose orientation was perpendicular to the plane of the epithelium and cuticle (Fig. 5C). These microtubules formed junctions with tonofibrillae of the ML in tissues not exposed to 0°C. Although microtubules in mammalian cells can dissociate at 0°C (Kirschner, 1978), electron micrographs showed

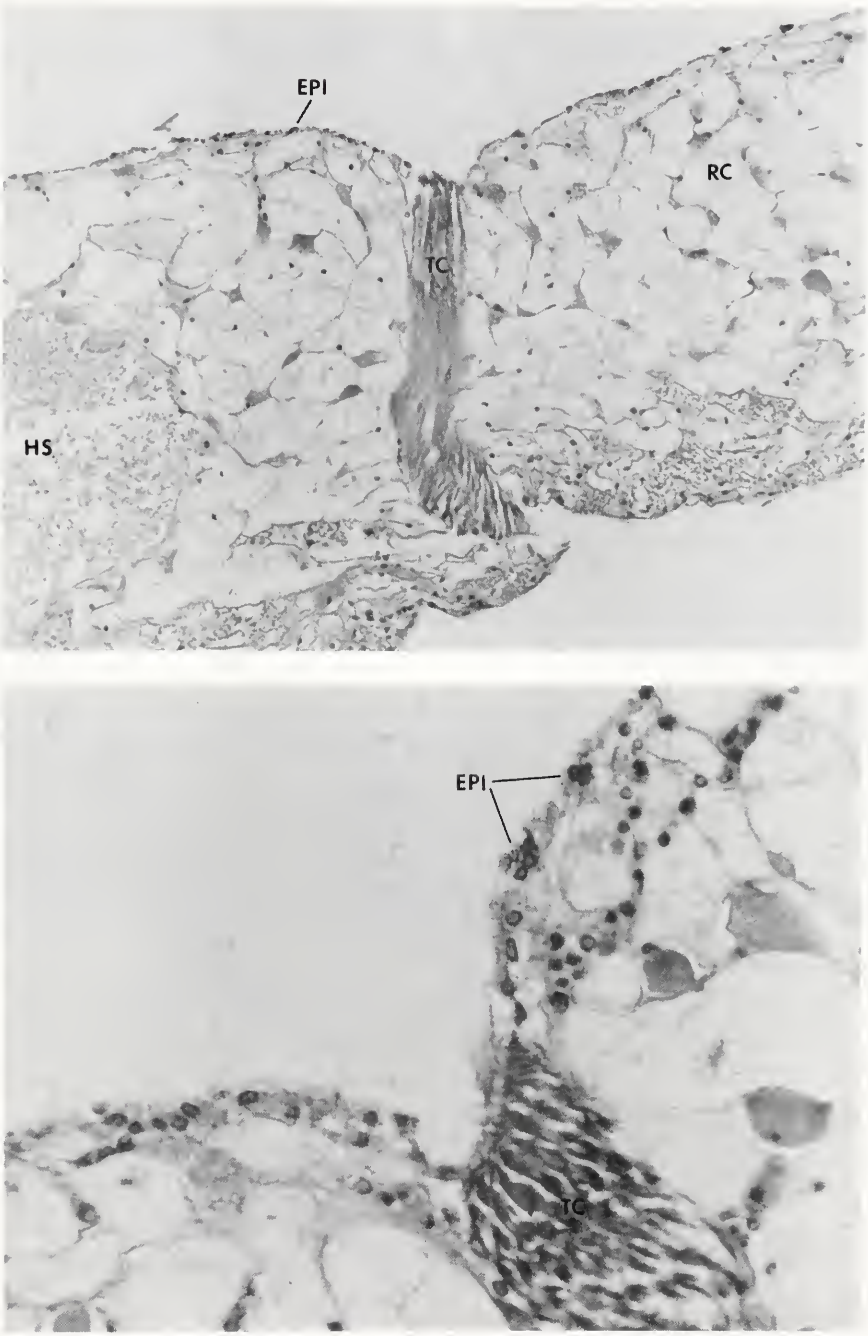

FIGURE 2. Transverse sections of integumentary tissues from branchiostegites that did undergo cold-induced apolysis. (A) *G. lateralis* chilled 1.5 h *in vivo*, then tissues removed, ×167. (B) Tissues removed from *G. lateralis*, then chilled 1.5 h *in vitro*, ×425. EPI, epithelial tissue; HS, hemolymph sinus; TC, tendinal cells; RC, reserve cells.

TABLE I

Brachyurans in which cold-induced apolysis occurred

Family	Species
Cancridae	*Cancer antennarius*
Gecarcinidae	*Cardisoma guanhumi*
	Gecarcinus lateralis
	Gecarcinus ruricola
Grapsidae	*Pachygrapsus crassipes*
	Sesarma cinerum
Majidae	*Pugettia producta*
	Taliepus nuttallii
Ocypodidae	*Ocypode quadrata*
	Uca pugilator
Portunidae	*Callinectes sapidus*
Xanthidae	*Menippe mercenaria*
	Rhithropanopeus harrisii

that the microtubules in the tendinal cells following cold-induced apolysis were not in disarray (data not shown).

Cold-induced apolysis was observed in species of all seven brachyuran families tested (Table I). Although the integumentary tissues of most specimens separated from the carapace within one h, the response for *R. harrisii* required 4.5 h. If animals were submerged in the ice-water at the bottom of the container, cold-induced apolysis did not occur. In adult majid crabs, *P. producta,* complete separation of the integumentary tissues from the exoskeleton at the cardiac region of the carapace required about 30 min exposure to 0°C and at the branchiostegite region of the carapace about 45 min (Table II). None of the controls, including ganglionectomized crabs kept at room temperature for 3 h, underwent apolysis. The integumentary tissues of crayfish branchiostegites remained firmly attached to the ML and the assays were scored as negative. All crayfish were alive following 3.5 h in ice. Although lobster integumentary tissues remained attached to the ML after 4 h of chilling and the assays were scored as negative, one of us found that chilling lobster claws led to partial separation and facilitated the removal of muscle (Mykles, 1985; Mykles and Skinner, 1986).

Cold-induced apolysis occurred in *G. lateralis* in early proecdysis (D_0, Skinner, 1962) metecdysis, and anedcysis. No animals in stage A of metecdysis were tested, but in stage B, which occurs well before the formation of the membranous layer (Drach, 1939; Skinner, 1962), the integumentary tissue of the branchiostegites could be easily separated from the exoskeleton following exposure to 0°C. Cold-induced apolysis was also observed in adult *P. producta* and *T. nuttallii,* which were in terminal anecdysis (Tessier, 1935; Hartnoll, 1963; O'Brien, 1984).

DISCUSSION

Results were equivocal as to whether the plane of separation during cold-induced apolysis occurred between the epithelium and the superficial ML or between the epithelium and the underlying tissues of the integument. Some segments of ML from animals that had undergone cold-induced apolysis had no tissues attached (Fig. 3A, 5A), while other segments had epithelial cells and fibers from tendinal cells present (Figs. 3B, C). The same degradative enzymes may be active during physiological and cold-induced apolysis despite the presence of fragments of epithelial and/or tendinal

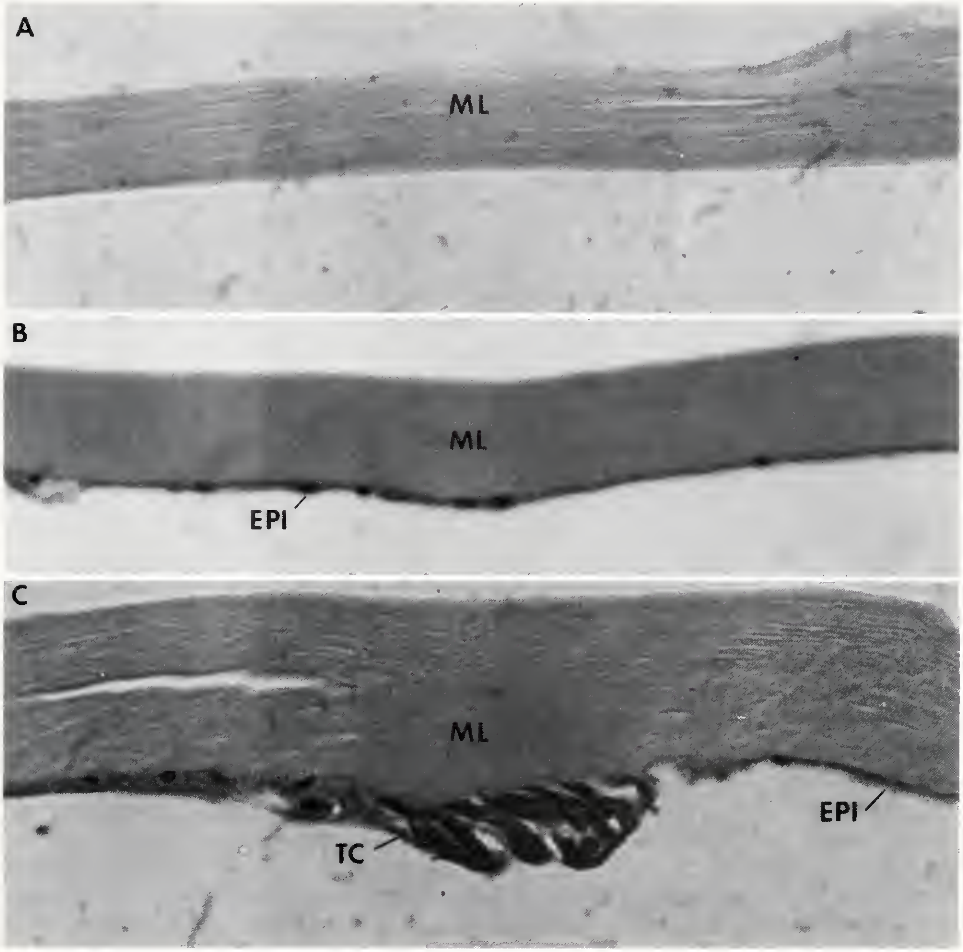

FIGURE 3. Transverse sections of ML from *G. lateralis* in (Fig. 1B) that did undergo cold-induced apolysis. Branchiostegites had been chilled for 1.5 h after removal from animal. Integumentary tissues and ML were then detached from each other and from the remainder of the exoskeleton, ×310. (A), (B), (C) Three areas of ML. Abbreviations as in Figure 2.

cells on some areas of ML following cold-induced apolysis; perhaps the incubation period was too short for complete separation to ensue.

Cold-induced apolysis occurred in tissues that had been isolated from crabs before exposure to 0°C (Fig. 2B, 3A–C) indicating that it is not a neural response. Electron micrographs of crab integumentary tissues indicated that cold shock did not cause the depolymerization of microtubules that can occur at 0°C in mammalian cells (Kirschner, 1978). Cold shock may have stimulated release into the apical extracellular environment of the integumentary tissues degradative enzymes responsible for apolysis. Such "apolysases" could have been stored within vesicles of epithelial cells (Koulish and Klepal, 1981) or in the several types of glands found in crustacean integumentary tissues (Skinner, 1962; Babu *et al.*, 1985). It is unlikely that cold-induced apolysis is due to lysosomal breakdown following cell death since it occurred within 1 h at 0°C but was not observed in dying animals even after 3 h at room

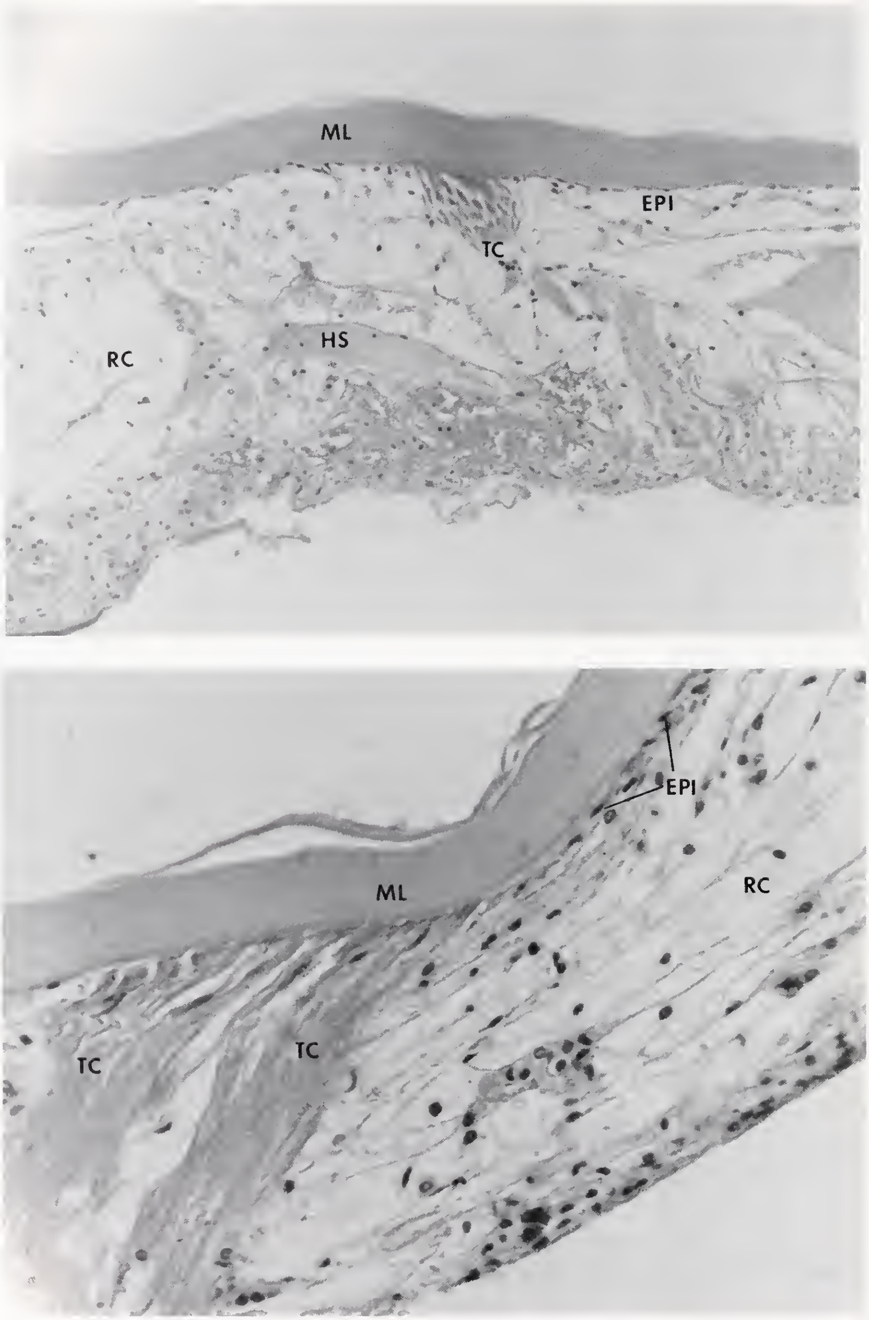

FIGURE 4. Transverse sections of integumentary tissues from branchiostegites from two *G. lateralis* that did not undergo cold-induced apolysis. (A) Untreated animal, ×167. (B) Tissues from ganglionectomized crab held at room temperature for 1.5 h, ×425. Abbreviations as in Figure 2.

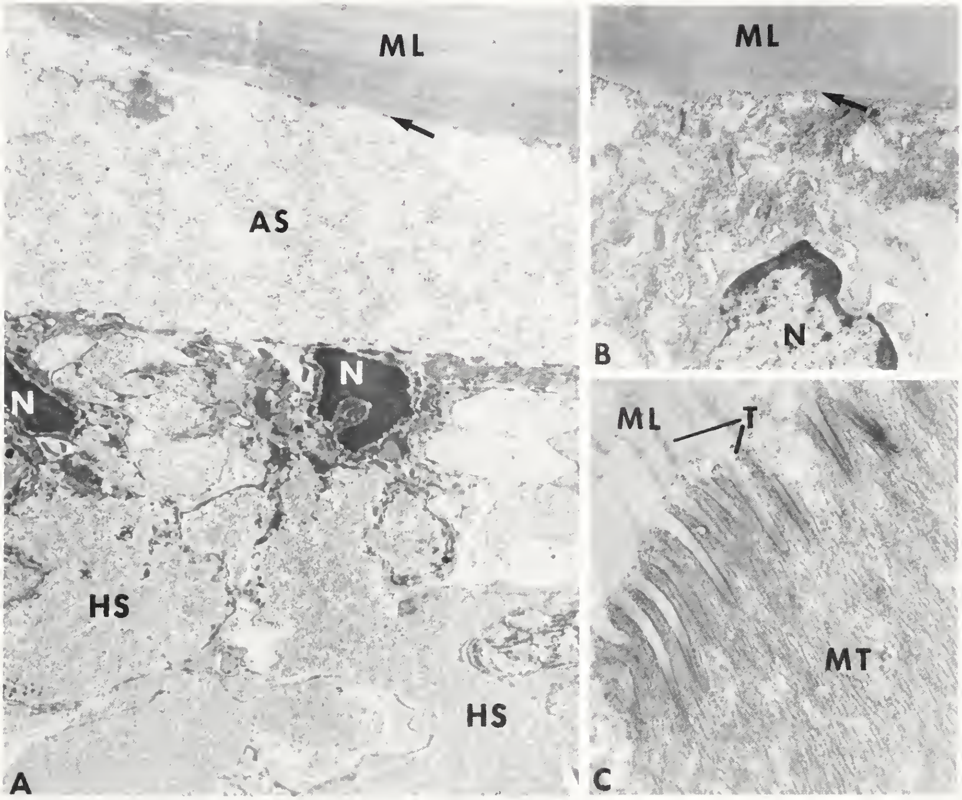

FIGURE 5. Electron micrographs of transverse sections of branchiostegite segments from two *G. lateralis.* (A) Crab chilled and then tissue removed. Epithelium completely separated from ML. No cellular material adhered to inner surface (arrow) of membranous layer, ×6700. (B and C) Tissues removed from untreated animal. (B) Epithelial cell attached (arrow) to ML, ×9600. (C) Junctions between microtubules (MT) of tendinal cell and tonofibrillae (T) of ML, ×20,000. AS, apolytic space; N, nuclei of epithelial cells; other abbreviations as in Figure 2.

temperature. It is also unlikely that cold-induced apolysis is caused by the release of cytoplasmic proteinases following rupture of cell membranes; freezing of cytoplasm occurs well below 0°C. Furthermore, cold-induced apolysis appeared to be localized near if not at the epithelial-cuticle interface with minimal disruption of underlying tissues removed from whole animals that had been chilled (Figs. 2A, 5A). After chilling, muscle fibers generated membrane potentials of −50 to −60 mv (unpub. obs.) suggesting that the surface membranes were intact. The induction of separation between the amuscular integumentary tissues and exoskeleton of the branchiostegites showed that cold-induced apolysis was not due to a reflexive contraction of muscle.

Localized differences in the length of time required to cause cold-induced apolysis may reflect the distribution and abundance of tendinal cells, which function to anchor muscle fibers and integumentary tissues to the exoskeleton. Tendinal cells are attached to tonofibrillae within the exoskeleton via specialized junctions (Green and Neff, 1972; Koulish and Klepal, 1981). At sites where muscles insert into the cuticle, such as the chelae, large numbers of such attachments would be detached as cold-induced apolysis occurred. The cardiac and branchiostegite regions, with many fewer muscle insertions, require less time to undergo cold-induced apolysis. Differences in

TABLE II

Length of time specimens of Pugettia producta *were chilled before separation of the ML from underlying tissues*

Time (min)	Separation of membranous layer from			
	Stomach muscle		Branchiostegite integument	
	0°C	23°C	0°C	23°C
0	–	–	–	–
10	–	–	–	–
30	+	–	–	–
45	+	–	+	–
60	+	–	+	–

+ indicates separation occurred; – indicates separation did not occur.

the distribution of tendinal cells may also explain why apolysis does not occur uniformly over the entire surface of the proecdysial animal (Drach, 1939; Koulish and Klepel, 1981).

Degradation of the crustacean exoskeleton during proecdysis involves the secretion of chitinases, chitobiases, and proteinases by the underlying integumentary tissues and is thought to be regulated by the molting hormone 20-OH ecdysone. Whether any of these enzymes are activated or newly synthesized in response to chilling is not known. Activity of crustacean degradative enzymes has been observed at 0°C; extensive lipolysis in three species of krill either maintained at 0°C or frozen (Saether *et al.,* 1986) was attributed to post mortem proteolysis (Saether *et al.,*1986 citing the Ph.D. thesis of Ellingsen, 1982). Since dying (ganglionectomized) *G. lateralis* did not undergo apolysis when kept at room temperature for 3 h, cell lysis cannot account for the phenomenon we describe.

Many crustaceans exhibit indeterminate growth, molting throughout their lives (Hartnoll, 1982). For example, even very large specimens of *G. lateralis* enter proecdysis following autotomy of a critical number of limbs (Skinner and Graham, 1970; Holland and Skinner, 1976). We observed that metecdysial and anecdysial *G. lateralis* did undergo cold-induced apolysis. The simplest interpretation, assuming that the same degradative enzymes are involved in both proecdysial apolysis and cold-induced apolysis, would be that the relevant enzymes are synthesized during metecdysis. However, the ability of crabs that exhibit determinate growth to undergo cold-induced apolysis requires a modification of this explanation. With the exception of males of the genus *Chionoecetes* (Watson, 1970), adult crabs in the family Majidae (Tessier, 1935; Hartnoll, 1963; O'Brien, 1984) are like adult *Leuroleberis zealandica* (Ostracoda) (Fenwick, 1984) and adult copepods (Hartnoll, 1982); they do not molt again. Cold-induced apolysis in adult majids suggests that at least some of the enzymes involved in apolysis may not be synthesized during or immediately prior to the proecdysial period. They may be constitutive in the integumentary tissues of some brachyurans. This could also account for the presence of chitinase activity in integumentary tissues of crabs in all stages of the molt cycle (Jeuniaux, 1959).

We suggest that mechanisms similar to those responsible for cold-induced apolysis may cause the separation of tissues from the exoskeleton of crabs bitten by an octopus. Although crab prey were separated from *Octopus* predators immediately after paralysis was induced by a single bite, the tissues of the crabs could be separated intact from the exoskeleton within 30 min (Nixon, 1984). Nixon postulated that the octopus injected enzymes highly specific for the arthrodial membrane and musculoskeletal attachments since, in another experiment, crab tissues were recognizable 2 h after ingestion by the octopus. Our data suggest that instead of exogenous proteinases, the octopus may inject substances that stimulated crab integumentary tissues to synthesize, activate, or secrete degradative enzymes.

Cold-induced apolysis might influence conclusions drawn from some growth studies. The growth rate of populations of crustaceans is sometimes estimated from the percentages of animals in proecdysis and metecdysis (Hartnoll, 1982). Because apolysis is such an easily recognizable characteristic, any crustacean with an exoskeleton separated from the underlying tissues is likely to be classified as proecdysial without further inspection. Our data indicate that, at least for some Brachyura, if individuals in the metecdysial and anecdysial stages are kept on ice for extended periods of time before examination, they may be incorrectly judged to be in proecdysis.

The inability of the two astacurans to undergo cold-induced apolysis is not surprising given their exposure to 0°C and below in their natural habitats (Squires, 1970; Becker *et al.*, 1977; Mirenda and Dimock, 1985). *R. harrisii*, an estuarine crab, is also exposed to low temperatures during the winter. Turoboyski (1973) reported that a European subspecies of *R. harrisii* survived exposure to ice for short periods, in agreement with our observation that cold-induced apolysis in *R. harrisii* required a longer exposure to ice than the other brachyurans tested. The occurrence of apolysis after chilling in *R. harrisii* emphasizes the fact that the phenomenon is not limited to tropical or even subtropical crabs. We observed cold-induced apolysis in a number of temperate species that tolerate low temperatures (Table I). *Callinectes sapidus* is commonly found at Cape Cod and in the North Sea (Williams, 1984) while the ranges of *Cancer antennarius* and *Pachygrapsus crassipes* extend northward to Oregon (Morris *et al.*, 1980). *Pugettia producta*, which underwent cold-induced apolysis in only 45 min (Table II), is found at even higher latitudes in the cold waters off British Columbia and Alaska (Morris *et al.*, 1980).

The mechanism that causes cold-induced apolysis may have been modified by selective pressure in crustaceans that tolerate 0°C. This possibility is strengthened by preliminary results that demonstrate the presence of alkaline cysteine proteinase activity in extracts of integumentary tissues of *G. lateralis* but not in those of lobster (O'Brien and Skinner, 1985), a species that did not undergo cold-induced apolysis.

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A NEW TRICHOMYCETE COMMENSAL WITH A GALATHEID SQUAT LOBSTER FROM DEEP-SEA HYDROTHERMAL VENTS

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ABSTRACT

A new species of eccrinid fungus (Trichomycetes) is described. The fungus is a commensal in the cardiac and pyloric stomachs of the galatheid squat lobster *Munidopsis subsquamosa* (Decapoda, Anomura) collected from deep-sea (2600 m) hydrothermal vents. This is the first record of a trichomycete from depths greater than 50 m. About 40% of the galatheids examined were heavily infested; this proportion varied among the subsamples collected from various vent sites. No fungal thalli were observed in the smallest galatheids.

INTRODUCTION

The trichomycetes belong to a class of fungi which is obligately associated with living arthropod hosts. The fungal thalli, attached to the cuticular lining of the foregut and/or hindgut of their host by a holdfast, do not invade the host tissues. Lichtwardt (1976, 1986) summarizes the biology and systematics of the four orders of trichomycetes, including their distribution among the various arthropod groups. Members of the order Eccrinales, characterized by an unbranched, non-septate thallus, are known from diverse arthropods, including insects, millipeds, isopods, amphipods, and anomuran and brachyuran crabs.

The discovery of a new species of eccrinid fungus was the consequence of a routine analysis of gut contents of the galatheid squat lobster *Munidopsis subsquamosa* Henderson. The host galatheids, collected from hydrothermal vent sites in the Pacific Ocean, are thought to belong to a cosmopolitan species complex distributed throughout the world's oceans (J. Ambler, pers. comm.). In addition to regular diet items such as small limpets, crab larvae, polychaetes, and folliculinid protozoans, many of the galatheid stomachs contained long, thin filaments attached to the stomach wall. A superficial resemblance to the filamentous bacterium *Beggiatoa,* known to occur at vent sites and to be grazed by galatheids (Hessler and Smithey, 1983), was noted. Upon further examination, however, the filaments were identified as a new species of eccrinid fungus. This is the first record of a trichomycete from abyssal depths.

MATERIALS AND METHODS

The host galatheids were collected from several different sites during expeditions to Galapagos Rift and East Pacific Rise hydrothermal vents (see Hessler and Smithey, 1983, and Hessler *et al.,* 1985, for descriptions of vent sites). The galatheids forage over the hard, rocky bottom and among the clumps of mussels and clams in the vicinity of the venting water. They were collected inadvertently by the submersible ALVIN as it maneuvered around vent areas, and were subsequently carried to the

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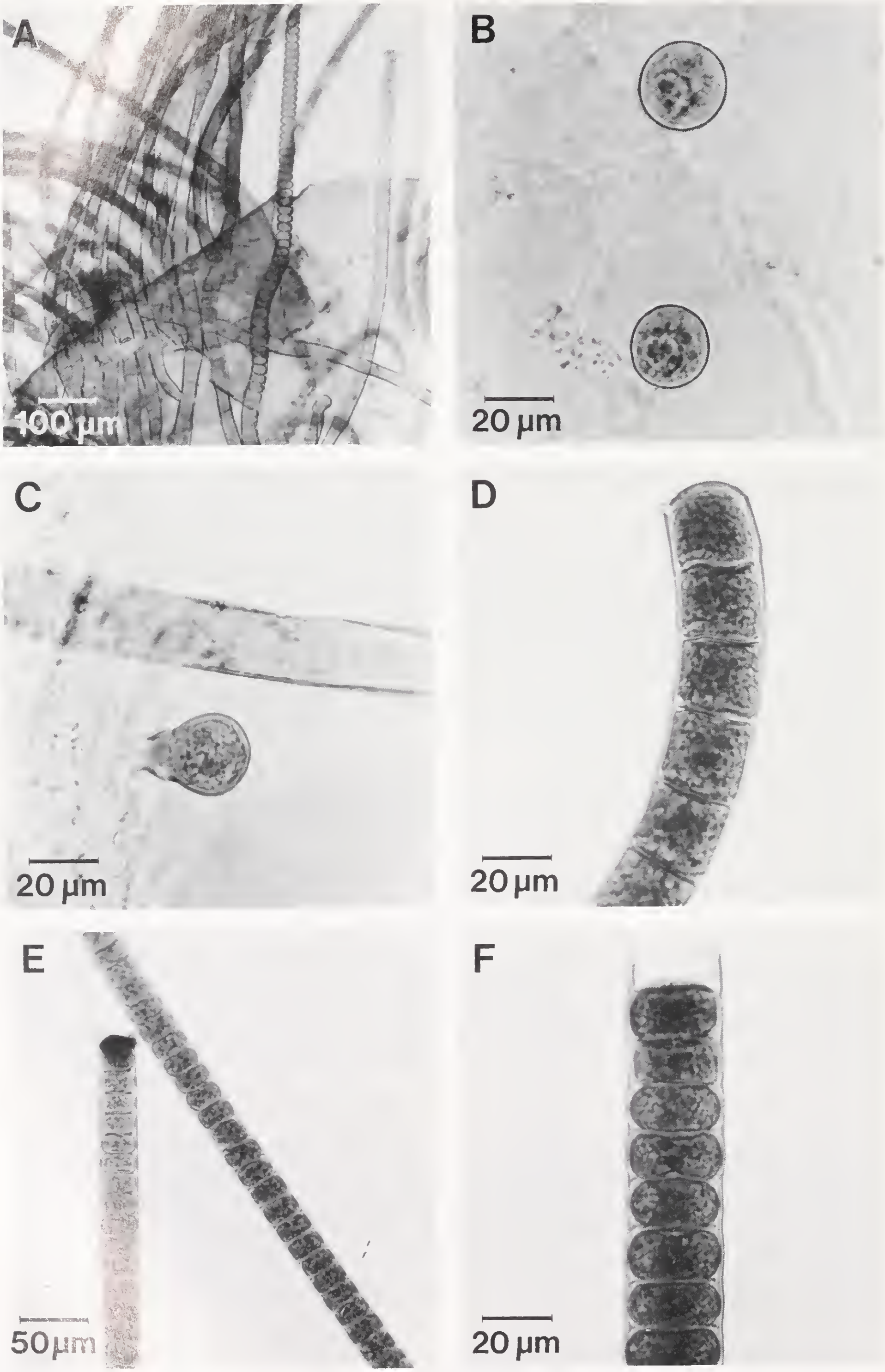
A
100 µm
B
20 µm
C
20 µm
D
20 µm
E
50µm
F
20 µm

surface. Most of the animals were preserved in formalin, then stored in alcohol. The stomachs of four specimens were dissected and preserved in buffered glutaraldehyde. The fungal type specimen was obtained from one of these stomachs. Slides of this stomach were prepared by mounting pieces of material in lactophenol cotton blue, and were examined by light microscopy.

DESCRIPTION

Arundinula abyssicola Van Dover and Lichtwardt, sp. nov.

Thalli ventriculum Munidopsis subsquamosae (Decapodium Anomurium) ad 2 mm longi, 24–27 μm crassi. Sporangiosporae primae infestationis 12–17 μm longae, 28–38 μm crassae, sub liberatione paene sphaericae factae, 22–27 μm diametro, 4–8-nucleatae, infestationis secundariae 17–24 μm longae, 22–27 μm crassae, item 4–8-nucleatae.

Holotype (glutaraldehyde-preserved stomach) GAL-1, deposited with R. W. Lichtwardt, University of Kansas. Type locality and specimen: Attached to cardiac and pyloric stomach cuticle of *Munidopsis subsquamosa* Henderson; "Rose Garden" hydrothermal vent site at 00°48.0'N, 86°09.9'W (Galapagos Rift), 2600 m.

Unbranched thalli usually not exceeding 2 mm in length, 24–27 μm diameter, with sporulating tips occasionally up to 40 μm diameter. Attached by a centric basal holdfast 10–20 μm long × 18–22 μm diameter to the cardiac and pyloric stomach of *Munidopsis subsquamosa.* Primary infestation sporangia produced in very long series, their single sporangiospores ellipsoidal but flattened in side view, 12–17 μm long × 28–38 μm diameter. Mature spores emerge successively through the end wall of the adjacent empty sporangium, becoming almost spherical after release, 22–27 μm diameter and 4–8 nucleate. Secondary infestation sporangiospores 17–24 μm long × 22–27 μm diameter, 4–8 nucleate. Spore mother-cells persistent and usually becoming fertile.

RESULTS

Some of the stomachs we examined contained possibly one or two thousand thalli, many sporulating prolifically (Fig. 1A). Each sporangium produces a single spore. The primary infestation sporangiospores (Figs. 1B, C) function to disseminate the fungus from one individual anomuran to another after passage from the gut, whereas secondary infestation sporangiospores (Fig. 1D) are capable of germination within the same gut where they are produced, and therefore serve to increase the amount of infestation endogenously.

In the specimens we examined critically, thalli with primary spores were more numerous. After the emergence of a mature (terminal) primary spore, the walls of the vacated sporangia decompose, apparently with the aid of attached bacteria (Fig.

FIGURE 1. A. Thalli in various stages of development attached to a piece of cuticle removed from the host stomach. B. Top view of two primary infestation sporangiospores attached to the stomach lining by means of a rudimentary secreted holdfast that can be seen indistinctly through the spores on their under surfaces. C. Side view of a primary infestation sporangiospore attached to the cuticle by a small holdfast and beginning to germinate. D. Secondary infestation sporangiospores seen developing inside their individual sporangia. E. Thalli that have produced primary infestation sporangiospores. Note the dense bacteria which assist in the decomposition of the sporangial walls after release of spores at the tip of one thallus. F. Thallus tip with primary infestation sporangiospores, the most terminal one beginning to emerge through the end wall of the adjacent empty sporangium.

1E). The production of primary and secondary spores is basipetal, such that the thalli become shorter as the older, terminal spores are released one by one and the empty sporangia degenerate (Fig. 1F).

The tips of thalli producing secondary spores are generally narrower than thalli producing primary spores, and the secondary sporangia can be identified by their square to slightly rectangular appearance in side view. Release of the more or less cylindrical secondary spores occurs most often through a pore or tear in the lateral wall of the sporangium. In some instances we observed rows of several thalli that probably originated from adjacent spores in sporangia lying against the cuticle of the stomach.

The development of thalli from both primary and secondary spores is similar. The spore first attaches to the cuticle by means of a rudimentary secreted holdfast, and germination takes place between this point of attachment and the spore body. As growth of the non-septate, coenocytic thallus continues and more holdfast material is being secreted, the germinated spore, now called a spore mother-cell, persists apically throughout the growth of the thallus (Fig. 2A). After it becomes separated from the main thallus by formation of a cross-wall, the spore mother-cell eventually becomes the terminal sporangium (Fig. 2B). In many other eccrinid genera the spore mother-cell disintegrates before the thallus reaches maturity.

It is possible in *A. abyssicola* to distinguish those thalli that originated from primary spores from those produced by secondary spores, so long as the spore mother-cell is still present. Primary spore mother-cells are positioned at the tip of the thallus in line with the axis, and are rounded to sometimes flattened at the apical end (Figs. 2A, B), whereas secondary spore mother-cells tend to be more angular and have a reflexed position due to the area on the spore wall where attachment and germination took place (Fig. 2C).

We did not find thalli of *A. abyssicola* attached to the hindgut lining of *M. subsquamosa*, a condition reported to occur in other species of *Arundinula* except for *A. galathea* (also commensal in a galatheid squat lobster). Nor did we find the oval to ellipsoidal sporangiospores with single appendages at each pole that have been seen, though rarely, in the exuviae of the respective anomuran hosts of *A. capitata* and *A. washingtoniensis*.

Arundinula abyssicola is distinct from the other five valid species of the genus on the basis of the shape and dimensions of its spores and its unusual anomuran host. It also can be distinguished from most other species of *Arundinula* by the morphology of its thalli and the shape and location of its holdfast (Fig. 2D). Morphological characteristics of *Arundinula* species are summarized in Table I.

Discussion

The genus *Arundinula* includes five species in addition to *A. abyssicola* described here. They are known to occur in several families of shallow-water marine anomurans as well as in freshwater crayfish (reviewed in Lichtwardt, 1986). The basic morphology of the new eccrinid species is clearly allied with that of the other members of the genus, despite the extraordinary depths at which it was collected and the unusual physico-chemical environment of the hydrothermal vents.

Not all of the galatheids collected from the Galapagos Rift and the East Pacific Rise contained fungi (Table II). About 40% of the animals were infested. This proportion was the same in both male and female subpopulations (Table III). A greater proportion of gravid females were infested than non-gravid females.

No fungi were found in the smallest individuals (Table III). It may be that these

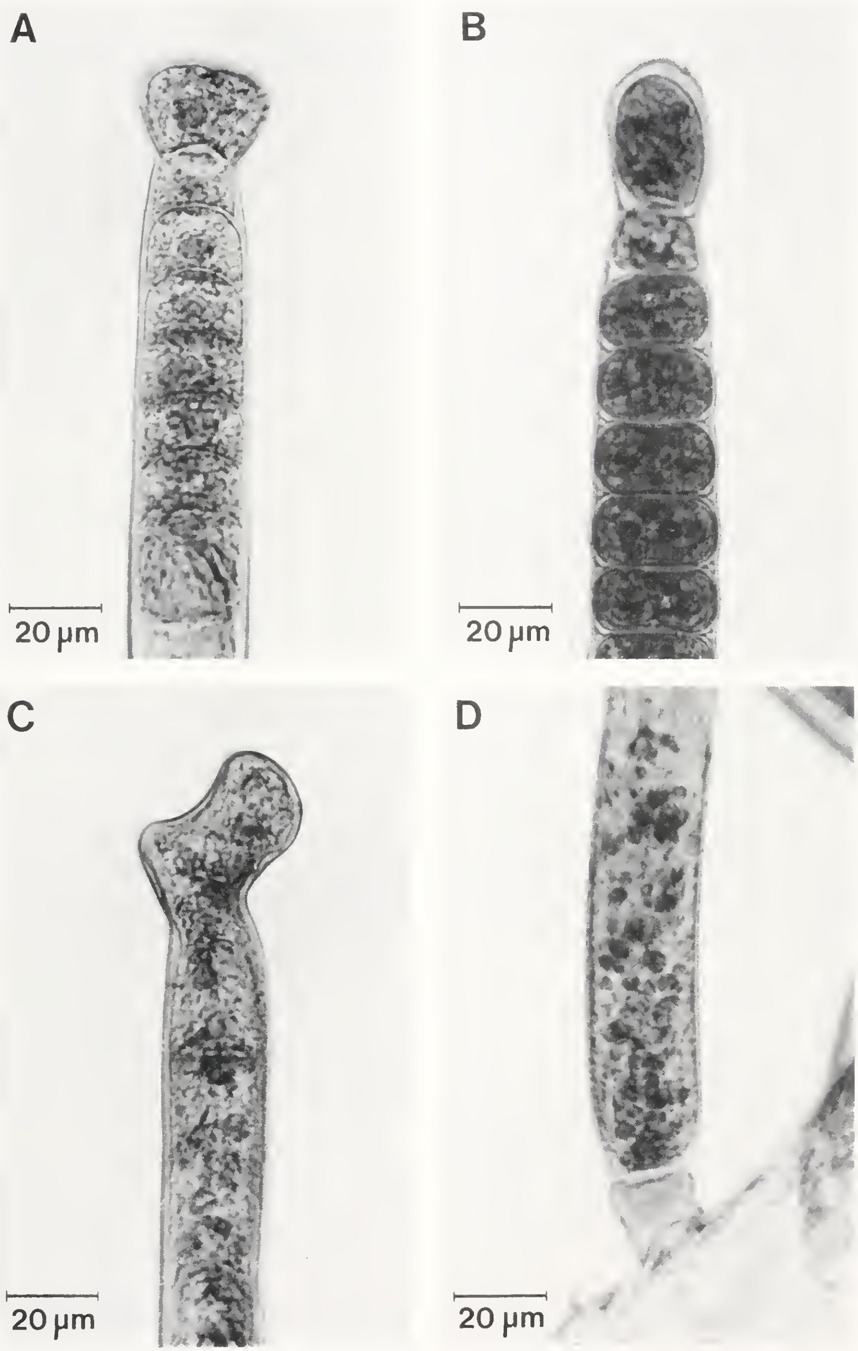

FIGURE 2. A. Tip of a thallus that developed from a primary infestation sporangiospore, beginning to form septa so as to produce sporangia. At this stage each resulting cell is uninucleate. B. A primary spore mother-cell that has become a sporangium and contains a single spore. C. Developing thallus tip with a reflexed secondary spore mother-cell. D. Holdfast anchoring a mature thallus to the stomach lining.

TABLE I

Morphological characteristics of Arundinula *species summarized from Lichtwardt (1986) and this study*

		Primary infestation sporangiospores		Secondary infestation sporangiospores		Thalli		
Species	Host	Shape	Size (μm)	Shape	Size (μm)	Form	Length (mm)	Diameter (μm)
A. capitata Léger & Duboscq, 1906	Paguridae (*Eupagurus*, *Pagurus*)	flat, disk-like	—	elongate	13–15(–30) (length)	Foregut: thick-walled Hindgut:	 1–2(–3) ≥10	 17–22, 23–30 9–17, 35–60
A. galatheae Manier & Ormières ex Manier, 1968	Galatheidae (*Galathea*)	round or oval	27–31	oval	45–65 × 30–45	Rigid, thick-walled; tapered	≤3	27–47 base, 50–83 apex
A. haplogaster Hibbits, 1978	Lithodidae (*Haplogaster*)	oval	20–35(–43) × 5–7.5	dolioform	20–35 × 17–35	Foregut and anterior part of hindgut: a) curved b) straight Hindgut: a) undulate b) coarse, tapered c) tapered d) thick-walled	 3 — 6 15 7 8	 16–35 — 12–25 41–50 base 17–25 apex 37–43 base, 16–21 apex 55–105 base, 9 apex
A. orconectis Lichtwardt, 1962	Astacidae (*Orconectis*)	spherical	24–34	swollen proximally	36 × 20	Thin-walled	up to 20	20
A. washingtoniensis Hibbits, 1978	Paguridae (*Paguristes*)	ellipsoidal with long (120 μm), tapering appendage at each pole	—	dolioform	various: 20–35 × 10–20 40–80 × 14–19 135–390 × 11–20	Foregut: a) thick-walled b) thick-walled c) very-thick-walled Hindgut: a) thick-walled b) thick-walled	 1.8 4.2 .5–.6 6.5 13.5	 22–45 17–27 60–120 20–45 11–30
A. abyssicola sp. nov.	Galatheidae (*Munidopsis*)	spherical	22–27	cylindrical	17–24 × 22–27	Thin-walled	<2	24–27, sporulating tips up to 40

TABLE II

Eccrinid infestations of galatheid populations at hydrothermal vent sites in the eastern Pacific Ocean

	Year	# Infestations/ # *M. subsquamosa* examined	% Infested
Galapagos Rift (00°48.0′N; 86°09.0′W)			
Garden of Eden	1979	1/3	33%
Mussel Bed	1979	0/1	0%
Rose Garden	1979	0/1	0%
Rose Garden	1985	4/5	80%
East Pacific Rise (20°50.0′N; 109°06.0′W)			
Clam Acres	1982	8/23	35%
Clam Acres	1985	1/4	25%
TOTAL		14/37	38%

individuals are molting so frequently that the fungus is shed with the cuticular lining of the stomach more rapidly than it can be re-acquired, or that the animals simply have not had a chance to be exposed to the fungus at all.

The Rose Garden site in 1985 seems to have a higher proportion of infestations relative to other sites (Table II). Greater densities of galatheids occurred at Rose Garden in 1985 than in 1979 (R. Hessler, pers. comm.); large host populations may facilitate fungal infestations. The more sparse populations of non-vent deep-sea galatheids may be expected to have a very low incidence of infestation. The age of a vent community and its galatheid population may also influence the incidence of infestation. At older vents, more time will have been available for transmission of the fungus throughout the local population.

The nature of the relationship between eccrinids and their host is not clear. The early literature referred to the fungi as parasites, but neither detrimental nor beneficial effects by eccrinids have been demonstrated. The fungus and host are not symbiotic in the sense of the symbiosis between other vent organisms such as the clams, mussels,

TABLE III

Eccrinid infestations of M. subsquamosa *by host sex and size*

	# Infestations/ # *M. subsquamosa* examined	% Infested
Sex		
Males	4/10	40%
Females		
non-gravid	3/13	23%
gravid	6/10	60%
combined	9/23	39%
Size (rostral-carpace length)		
>50 mm	3/7	43%
40–50 mm	6/12	50%
30–40 mm	3/8	37%
<30 mm	0/4	0%

or vestimentiferans and chemoautotrophic bacteria. These vent animals are thought to derive much or all of their nutrition from their symbionts (reviewed in Cavanaugh, 1985), while the galatheid is known to be an active predator/scavenger. But it may be that subtle beneficial effects are present, such as the synthesis of complex nutrients by the fungus which might be used by their hosts when external nutrients are suboptimal.

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ABSTRACTS OF PAPERS PRESENTED AT THE GENERAL SCIENTIFIC MEETINGS OF THE MARINE BIOLOGICAL LABORATORY

AUGUST 19–21, 1986

Abstracts are arranged alphabetically by first author within the following categories: cell motility and cytoskeleton; developmental biology; ecology; fertilization; neurobiology; and physiology. Author and subject references will be found in the regular volume index in the December issue.

CELL MOTILITY AND CYTOSKELETON

Selective extraction of tubulin from dogfish erythrocyte cytoskeletons at low temperature. WILLIAM D. COHEN (Hunter College, New York, New York 10021).

Non-mammalian vertebrate erythrocytes have a distinctive cytoskeletal system consisting of a marginal band (MB) of microtubules, a cell surface-associated cytoskeleton (SAC), and, as demonstrated in some species, intermediate filaments. For studies of their cytoskeletal composition, dogfish (*Mustelus canis*) erythrocytes are particularly valuable because of large size, availability in quantity, and cold lability of the MB in living cells. Lysis of dogfish erythrocytes in 100 mM PIPES, 5 mM EGTA, 1 mM $MgCl_2$ (PEM) containing 0.6% Brij-58 produces "Brij cytoskeletons" which can be washed and resuspended repeatedly to remove soluble material. These cytoskeletons contain MBs as shown by indirect anti-tubulin immunofluorescence, and by release of intact MBs via elastase digestion of the SAC. Their major components as determined by SDS polyacrylamide gel electrophoresis are spectrin-like proteins in the Mr 220K–240K range, a complex of polypeptides in the tubulin region, and actin. When the Brij cytoskeletons are incubated in PEM at 0°C, the MB appears to disassemble selectively. The disassembly supernate contains the tubulin region complex of proteins, with no high molecular weight components co-migrating with brain MAP2 or myosin. Traces of actin and spectrin-like proteins, observed only when gels are overloaded, are probably SAC contaminants. Upon rewarming, the initially clear 0°C supernate becomes turbid due to formation of masses of fibrillar material visible in phase contrast (presumed microtubule aggregates). Selective extraction of the tubulin region polypeptides is low temperature dependent; it is enhanced in the presence of 0.1 M KCl, and appears to reach an end point after about 30 min under the conditions tested. Whether all of the tubulin-region proteins are in fact tubulin remains to be demonstrated. The Brij cytoskeletons can be stored in PEM containing 50% v/v glycerol at −20°C. These cytoskeletons are an excellent source of MB tubulin in milligram amounts, and should be of considerable value for future work on disassembly and reassembly of MB microtubules.

I thank my former students, Dr. Diana Bartelt and Dr. Jackie Joseph-Silverstein, who made initial observations on which this work was based. Supported by PSC-CUNY Grants 665142 and 666296, and by NSF PCM-8409159.

Rheology of extruded axoplasm: evidence for polymer sliding. EDWIN B. GEORGE AND RAYMOND J. LASEK (Case Western Reserve University Medical School).

Studies of the axonal transport of cytoskeletal proteins indicate that they are transported as polymers moving at different rates. This suggests that axonal polymers (microtubules and neurofilaments) must be able to slide relative to one another. We tested this by studying the stress-strain relationship of axoplasm extruded from the giant axon of *Loligo pealei.*

The axoplasm was mounted between two clamps, one attached to a pulling device (rates between 0.5 and 25.2 μm/s), and the second attached to a sensitive force transducer. The axoplasm was immersed in paraffin oil to prevent drying or extraction of diffusable proteins.

When stretched under these conditions, the axoplasm reached lengths up to seven times its original length, and increases in resting length by two to three fold were produced regularly. The stretching must cause a rearrangement of the polymers since they are not very elastic. The axonal polymers are relatively straight and closely aligned. This geometry requires that they either slide relative to one another or break.

When axoplasm was stretched at a constant rate, the force rose to constant level. If stretching was stopped, the force slowly decayed. Both the rising phase and decay could be described by a linear combination of exponential functions. These stress-strain relationships indicate a visco-elastic deformation, such as occurs in bulk solutions of uncross-linked polymers.

Repeated short stretches of a piece of axoplasm gave consistent force records. This shows the nature of the associations between the polymers was unaffected by deformation: any bonds that are broken during stretching quickly reform in the new configuration.

The forces generated ranged from 2 to 13 dynes/cm^2. If this force were distributed evenly, it could be generated by one dynein or myosin head for every 1000 μm of polymer. Thus, it is biologically feasible that forces could be generated within the axon which could permanently displace cytoskeletal polymers, as has been suggested by the model of polymer sliding during axonal transport.

E. B. George was supported during these investigations by a Grass Fellowship in Neurophysiology.

Heat stable fractions of squid giant nerve axoplasm do not contain tau or MAP2 immunoreactivity. KENNETH S. KOSIK AND JANIS METUZALS (Marine Biological Laboratory).

The identity and characterization of microtubule-associated proteins (MAPs) in the squid giant axon is incomplete. In the high molecular weight group a 260 Kd MAP designated axolinin and a 320 Kd MAP have been described. Neither are heat stable (Kobayashi *et al.* 1986, *J. Cell Biol.* **102:** 1699–1709). Because of our interest in the pathobiology of the MAPs, particularly as they become incorporated into the Alzheimer neurofibrillary tangle, we have endeavored to study the MAPs in the squid system which is more amenable to functional experiments. The MAPs implicated as constituents of the neurofibrillary tangle are tau and MAP2, which share the property of heat stability. These two proteins also may serve to define axonal and dendritic domains respectively in mammalian systems. We sought to determine whether the squid giant axon contains proteins that might be considered homologous with tau or MAP2 by the preliminary criteria of cross immunoreactivity or electrophoretic co-migration of the heat stable fraction from taxol purified microtubules. Highly specific polyclonal and monoclonal antibodies to tau and MAP2 failed to detect any immunoreactive proteins in total homogenates of squid axoplasm, sheath, and ganglion when individually analyzed by "Western" blots. The heat stable fraction from taxol purified microtubules contains two proteins at 60 Kd and 210 Kd and two low molecular weight bands below 20 Kd. These proteins appear only partially heat stable since they also appear in the heat labile precipitate. All of these bands can be readily detected when a high speed supernatant is made 0.75 *M* NaCl, boiled for 5 min, and the supernatant analyzed by SDS-PAGE. Whether these proteins are MAPs awaits morphological determination of their relationship to microtubules and their ability to cycle with tubulin.

Supported by NIH grants K07 NS00835 and R01 AG06172 and the Medical Research Council of Canada grant MA-1247.

Sidearms on squid axoplasmic vesicles and microtubules. GEORGE M. LANGFORD (University of North Carolina at Chapel Hill), DIETER G. WEISS, AND DIETER SEITZ-TUTTER.

Native microtubules dissociated from the bulk axoplasm of the squid giant axon glide and transport vesicles. We performed experiments designed to determine the ultrastructure of the crossbridges responsible for vesicle attachment and transport along microtubules. High resolution electron microscopic images of microtubules and vesicles obtained from negatively stained samples show that vesicles, 50 to 100 nm in diameter, are decorated with sidearms. The sidearms were attached to the surfaces of the anterogradely transported vesicles in the presence of ATP. These sidearms were made of one to three filaments of uniform diameter. Each filament measured 5–6 nm in width and 30–35 nm in length. The filaments in some of the sidearms had splayed apart by pivoting at their base thereby assuming a "V" shape. The spread configuration illustrated the independence of the individual filaments. The filaments in the other sidearms were closely spaced and oriented parallel to each other, a pattern called the compact configuration. In axoplasmic buffer containing AMP-PNP, structures indistinguishable from the filaments of the sidearms on the vesicles were observed attached to microtubules. Pairs of filaments, thought to represent the basic functional unit, were observed attached to adjacent protofilaments of the microtubules by their distal tips. These data support a model of vesicle movement in which a pair of filaments within a sidearm forms two crossbridges and moves a vesicle by "walking" along the protofilament of the microtubule.

Visualizing myosin in living cells. JOSEPH W. SANGER, BALRAJ MITTAL, JEFFREY S. DOME, AND JEAN M. SANGER (University of Pennsylvania).

Myosin light chains were prepared from chicken skeletal fast muscle by standard procedures and reacted with iodoacetamide rhodamine. PAGE showed three bands with approximate molecular weights of 21 kD, 19 kD, and 17 kD, all of which fluoresced when transfered to nitrocellulose and illuminated with UV light. The fluorescent light chains interacted with the free cross-bridge regions of the A-bands of isolated myofibrils producing a doublet of fluorescence in the center of the A-bands. In contrast, when microinjected into embryonic chick skeletal myotubes, the fluorescent light chains were incorporated along the complete length of the A-band with the exception of the center or pseudo-H region. Co-injection of fluorescein labeled alpha-actinin and rhodamine labeled light chains into myotubes produced complementary localization of the two proteins with alpha-actinin in the Z-bands and light chains in the A-bands. Living non-muscle cells incorporated the labeled myosin light chains into stress fibers and contractile rings. The banded pattern of incorporation of the myosin in stress fibers resembled myosin antibody stained cells. When the stress fibers of injected cells were disrupted with DMSO, fluorescently labeled myosin light chains were present in the cytoplasm but did not enter the nucleus. Removal of the DMSO led to the reformation of banded, fluorescent stress fibers within 45 minutes. Reformation of fluorescently labeled stress fibers also occurred following cytokinesis of cells that had been previously injected in interphase. In these cells a bright concentration of myosin was evident around the midbody as the stress fibers reformed. Our experiments demonstrate that fluorescently labeled myosin light chains from muscle can be incorporated readily into muscle and non-muscle myosins in living cells. The injected cells can disassemble and reassemble contractile fibers using a hybrid myosin molecule that contains muscle light chains and non-muscle heavy chains. It now should be possible to follow in many different living cells changes in localization and assembly of myosin, the motor protein of actin-myosin based contractile systems.

Actin-microtubule interactions in Allogromia *reticulopodia.* JEFFREY L. TRAVIS (Vassar College, Poughkeepsie, New York) AND SAMUEL S. BOWSER.

The motility of the *Allogromia* reticulopodial network is completely dependent on an intact microtubule system. The constituent microtubules move both axially and laterally through the pseudopodial cytoplasm, and these movements drive the pseudopodial movements. The characteristic bidirectional organelle transport occurs only along the microtubules. Disassembly of the microtubules completely inhibits all motility. Cytochalasin treatment does not inhibit microtubule movements or the microtubule-associated organelle transport. Cytochalasins do, however, cause the loss of pseudopod-substrate adhesion as well as the dramatic rearrangement of both the microtubule "cytoskeleton" and the pattern of motility. These results are interperted as indicating a microtubule-linked motor and an actin-based cytoskeleton. Rhodamine-phallodin staining localizes f-actin to discrete cytochalasin-sensitive plaques where the reticulopodia contact the substratum. Same-section correlative anti-actin immunofluorescence and electron microscopy show that the actin plaques at the cytoplasmic face of the ventral cell surface in the adhesion areas, and that a subset of the reticulopodial microtubules interact structurally with the actin filament-containing adhesion plaques. The combined data supports the idea that the actin plaques tether some of the reticulopodial microtubules to the ventral plasma membranes at the adhesion sites. This local restriction of microtubules is apparently necessary for morphogenesis and maintenance of the reticulopodia, and may allow the tension developed along the pseudopodial microtubules to be applied against the attachment point.

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DEVELOPMENTAL BIOLOGY

Early development of a polyclad flatworm: polar body formation, blebbing, and surface localization. JOHN M. ARNOLD (University of Hawaii), MICHAEL A. LANDOLFA, AND BARBARA C. BOYER.

The phylogenetic origins of mosaic development are still unexplained. Many molluscan and annelid species have polar lobes which exhibit cytoplasmic localizations of developmental significance. SEM observations may reflect similar localizations in the polyclad flatworm, *Hoploplana inquilina.*

Embryos were obtained by opening the gonads of two worms and mixing the gametes. Embryos were staged by meiotic events. Unfertilized eggs have a smooth surface with regularly spaced protruding granules. These disappear within 3 minutes after fertilization and at 40 minutes the egg surface is covered with microvilli and "microblebs." At 1¼ h first polar body extrusion begins and the zygote develops several large blebs which divide the egg into quadrants, one of which is initially unblebbed. The microvilli and "microblebs" disappear from this area and the surface becomes smooth. As meiosis proceeds the polar body becomes a club-shaped, then baton-shaped extension and more blebs appear as the larger blebs subdivide. Blebbing is confined mainly to the animal pole. Polar body extrusion and blebbing lasts about 10 minutes after which the zygotes again become spherical. Second polar body extrusion and a second less pronounced wave of blebbing occurs at about 2¼ h post-fertilization. As the second polar body becomes club-shaped the zygote again is divided into quadrants, one of which has a less prominent smooth area. The animal pole is more involved than the vegetal pole. First cleavage is unipolar and occurs about 4–4¼ h post fertilization. Blebs are formed in the furrow region, mainly in the animal hemisphere.

We conclude the following: (1) there is a definite pattern to the blebbing associated with the polar bodies; (2) most blebbing occurs in the animal hemisphere; (3) the blebbing pattern during second polar body formation is less pronounced than in first polar body formation but still divides the egg into quadrants, one of which appears smoother than the others.

Boyer (Boyer 1986, *Biol. Bull.* [these abstracts]) demonstrated that at the eight cell stage, macromeres will not develop into a larva but micromeres will undergo cellular differentation, albeit abnormal organogenesis. Uncleaved zygotes with vegetal portions removed usually develop normally. Therefore it appears that in polyclad flatworms a primative form of morphogenetic segregation occurs which may be reflected by the asymetrical blebbing pattern.

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Early development of a polyclad flatworm: development of partial eggs and embryos. BARBARA C. BOYER (Union College).

Experiments on uncleaved eggs and eight-cell embryos of the polyclad turbellarian *Hoploplana inquilina* examined cytoplasmic localization in this primitive spiralian. *In vitro* fertilized eggs were cut with glass needles to remove one-fourth to one-third of the vegetal region before the first meiotic division, between divisions and after the formation of the second polar body. In all experimental categories approximately two-thirds of the resulting larvae were normal while the remaining one-third exhibited lobe, tuft, and eye abnormalities.

Deletions of first quartet micromeres 1a or 1c *versus* 1b or 1d, two adjacent micromeres, three micromeres and isolation of the first quartet and macromeres resulted in increasingly abnormal larvae. Eighty percent of the larvae from single micromere deletions exhibited Muller's morphology. Deletion of 1a or 1c resulted in 57% two-eyed larvae while absence of 1b or 1d produced 74% two-eyed larvae. A significant increase in the number of one-eyed larvae occurred when two adjacent micromeres were deleted and a significant decrease in the number of larvae with Muller's morphology occurred with deletion of three micromeres. Isolated first quartets consistently formed spherical transparent larvae with multiple eyes (three or more in 77%). Isolated macromeres after deletion of the first quartet died in 81% of the cases and the surviving larvae typically were undifferentiated ovals with no eyes.

These results suggest that the cytoplasmic determinants in the *Hoploplana* egg usually are not localized vegetally, that eye and apical tuft determinants segregate to the micromeres, and that interactions between three micromeres and the macromeres are probably necessary for development of a Muller's larva with two eyes.

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H-ras oncogene DNA affects embryonic development in the sea urchin. DAVID DAVIDSON, SEYMOUR GARTE, AND WALTER TROLL (NYU Medical Center).

The embryonic development of the sea urchin *Arbacia punctulata* initially follows information provided by DNA from the ovum starting with the first division up to blastula stage. Replication of the newly formed sperm-egg combination of DNA may be a requirement for further differentiation leading to plutei formation. The activation of a specific DNA polymerase leading to selective DNA amplification (SDA) has been shown to involve a proteolytic step that is blocked by simple protease inhibitors *e.g.*, 6-amino caproic acid (6-ACA) and leupeptin (Heilbronn *et al.* 1985, *Int. J. Cancer* **36**: 85–91). We have found recently that the same inhibitors also block expression of H-ras oncogene transfection into NIH 3T3 cells presumably by inhibiting SDA (Garte, Currie, and Troll, manuscript submitted). We now show that in the sea urchin addition of 6-ACA given immediately or seven hours after ferilization and of leupeptin seven hours after fertilization prevented pluteus formation. This finding supports the view that protease inhibitors blocked the DNA replication necessary for pluteus formation. Next we tested whether H-ras oncogene DNA derived from human bladder cancer (p-EJ) was active in pluteus developement. DNA was introduced by a standard calcium phosphate transfection technique used in the tissue culture system. DNA/calcium phosphate was added to sperm just prior to mixing with eggs. Fertilized eggs treated with calcium phosphate (control) failed to develop into plutei. Those treated with H-ras oncogene DNA calcium phosphate formed plutei in a H-ras dose related manner. Moreover, the plutei formation due to oncogene DNA was blocked by the protease inhibitor 6-ACA. Thus the H-ras oncogene present in a variety of species also may participate in the development of the sea urchin.

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Sciences, and Special Institutional Grant no. 00009 from the American Cancer Society. D.D. was supported by the honors programm of N.Y.U. Medical School.

Sea urchin blastomeres are not dye-coupled during early embryogenesis. WILLIAM C. FORRESTER (University of Washington, Seattle, SM-30, Seattle, Washington 98195) AND JACEK KUBIAK.

Intercellular communication among sea urchin blastomeres was studied using microinjected fluorescent dyes. Fluoresceinated dextran (av. m.w. 110 kD) and Lucifer yellow (m.w. 430) were separately injected into intact fertilized eggs. The fluorescent blastomeres were disaggregated at the 16 cell stage after culturing the embryos in calcium-free seawater. Reaggregating individual fluorescent micromeres and unlabeled macromeres, mesomeres, or micromeres from similarly staged embryos yielded tight cellular morulae upon subsequent divisions. However, at no stage during this reaggregation was there any dye coupling between any of the blastomere combinations.

We may conclude, from our reaggregation experiments that large amounts of (micromere) cytoplasmic material is not transferred. Because the dyes become bound to cytoplasmic components and are thereafter unable to diffuse freely (after *ca.* 30 min.) (Stewart 1978, *Cell* **14:** 741–759) these experiments do not address the possibility of small membrane (gap-like) junctions between blastomeres. Therefore, we injected Lucifer yellow directly into blastomeres at the 2, 4, 8, 16, 32, and 64 cell stages. In no case was coupling observed. Culturing these injected embryos produced blastulae with dye sectors corresponding to the lineage of the originally injected blastomere, *i.e.*, if injection was into one blastomere at the 2 cell stage, precisely ½ of the blastula was labeled.

Previous studies also attempted to show how micromeres communicate with other blastomeres. Iontophoretic injections of Lucifer yellow directly into the micromeres (Woodruff *et al.* 1982, *Biol. Bull.*) and the fate of isotopically labeled micromere RNAs (Speigel and Rubinstein 1972, *Exp. Cell Res.* **70:** 423–430) did not demonstrate a direct cytoplasmic link between the micromeres and the other blastomeres.

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Steady currents enter wounds in the enveloping layer of Fundulus *embryos.* L. F. JAFFE, R. D. FINK, AND J. P. TRINKAUS (Marine Biological Laboratory).

Deep cells in *Fundulus* embryos move about between the extraembryonic region of the enveloping layer—a monolayered epithelium—and the surface membrane of the underlying yolk syncytial layer. When the enveloping layer is wounded, deep cells immediately begin to move toward the wound from distances up to 700 μm. This eventually results in a large accumulation of cells at the wound site. (Fink and Trinkaus 1986, *J. Cell Biol.*, abstract in press.) Electrical measurements on wounds in guinea pig skin have revealed large steady outward currents. These in turn generate large voltage gradients which could help direct cell movements closing the wound. (Barker *et al.* 1982, *Am. J. Physiol.* **242:** R358). Therefore we explored possible wound currents in a system where migratory cell responses can be directly observed.

Stage 21 *Fundulus* embryos were manually dechorionated, immobilized in 2% 1500 cps methyl cellulose in 2× Holtfreter's solution, and wounded by puncturing the enveloping layer and the yolk syncytial layer with a tungsten needle. Measurements of external wound currents were made with a vibrating probe both with the needle in place, and soon after withdrawing the needle.

We consistently observed large steady currents *entering* the wound. Current densities were often as high as 30 $\mu A/cm^2$ at the closest measurable point (about 30 μm from the wound). These densities fell off steadily with distance from the wound, reaching a half-maximum level about 100 μm away. When the needle was removed, the measured currents fell greatly in the course of five minutes, presumably indicating epithelial wound closure.

We were surprised to discover inward wound currents, since almost all epithelia normally pump positive charge inwards and are therefore expected to leak positive charge out through coarse wounds. Perhaps the wounded *Fundulus* enveloping layer pumps chloride or bicarbonate anions inwards to give the observed inward (positive) wound currents.

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Analysis of the meiotic cycles occurring in the absence of protein synthesis in activated Spisula *oocytes.* FRANK LUCA, TIM HUNT, AND JOAN RUDERMAN (Departments of Anatomy and Zoology, Duke University, Durham, North Carolina 27706).

When *Spisula* oocytes are parthenogenetically activated by adding KCl to 40 mM excess, they synchronously undergo germinal vesicle breakdown (GVBD), meiosis I, and meiosis II with the same timing as oocytes that have been activated by fertilization. Chromosome clustering, metaphase plate formation, and anaphase are easily identified by staining chromosomes with lacto-orcein or with the DNA-binding dye Hoechst 33342. When oocytes are activated in 0.1 *M* emetine, a potent inhibitor of protein synthesis, they undergo GVBD and meiosis I but fail to carry out meiosis II: the chromosomes remain condensed but do not align in a metaphase plate configuration or execute anaphase. Instead, the chromosomes lie close to the cell surface near the first polar body and remain in one or more clusters. Possible explanations include failure to form spindle, failure to replicate the centrosomes resulting in formation of a half-spindle or monaster, or failure of the microtubules to attach to the chromosomes.

To test these possibilities, we lightly extracted activated oocytes in a microtubule stabilizing buffer containing NP-40 followed by methanol/EGTA fixation and immunofluorescent staining with an anti-tubulin antibody. At the time of meiosis II in controls, emetine-treated cells showed smaller, definately bipolar, spindles but disorganized chromosomes. There were no obvious connections between the spindle and the chromosomes. All polymerized microtubules in control cells were associated with the meiosis II spindle, whereas emetine-cells showed many cytoplasmic microtubules. After second polar body formation in controls, a monaster formed nearby and became the single microtubule organizing center (MTOC). Emetine-treated cells formed one major aster (sometimes two) plus many smaller MTOCs which intensified with time, eventually packing the cell.

Communication compartments in ascidian embryos at the blastopore stage. M. W. MERRITT, J. A. KNIER, AND M. V. L. BENNETT (Marine Biological Laboratory).

In ascidian embryos, cells of the presumptive nervous system require contact with either chordal or anterior endodermal cells to develop. The required interaction may occur during gastrulation after the 64-cell stage. We examined cell communication in blastopore stage ascidian embryos by injecting low molecular weight dyes into cells identified by reference to fate maps of Conklin and others. Cells from embryos of two ascidians, *Ciona intestinalis* and *Phallusia mammillata* (generously supplied by J. Speksnyder and C. Sardet), were injected with Lucifer yellow (m.w. 450), 6-carboxyfluorescein (m.w. 376) or a combination of Lucifer yellow CH and rhodamine dextran (m.w. 10,000). In early gastrulation, just after the 64-cell stage, low m.w. dye injected into midline neural plate cells spread preferentially to the other neural plate cells, with much reduced spread to anterior ectodermal cells. In some cases, usually in slightly more advanced embryos, dye transfer between neural plate and notochord cells was observed. Prior to gastrulation, at the 64-cell stage, dye coupling was more widespread. Evidently, the observed dye coupling was mediated by gap junctions rather than cell bridges, because both Lucifer yellow CH and 6-carboxyfluorescein spread between cells, but rhodamine dextran always remained within the injected cell. These data suggest that the neural plate cells comprise a communication compartment, although not one completely isolated from ectodermal cells. A stage of communication with chordal cells may occur. Gap junctional conductance in ascidian embryos is steeply voltage dependent (see abstract by J. A. Knier), and egg cells of at least one species can exhibit two different resting potentials. Voltage dependence of coupling may mediate formation of communication compartments in these embryos.

M.W.M. is the recipient of a Reynolds Scholarship.

Equalization of first cleavage in Ilyanassa obsoleta *embryos.* JOANN RENDER (Hamilton College, Clinton, New York).

Polar lobes at first and second cleavage in the *Ilyanassa obsoleta* embryo shunt vegetal material to the larger CD and D blastomeres, respectively, resulting in unequal cleavage. Removal of the first cleavage polar lobe yields a veliger larva lacking lobe-dependent structures: eyes, statocysts, external shell, foot, and operculum. Embryos were immersed in 1.0–1.5 μg/ml cytochalasin B (CB) or 20.0 μg/ml sodium dodecyl sulfate (SDS) from the time of second meiotic polar lobe resorption until first cleavage. In these solutions polar lobes failed to form, resulting in equal first cleavage. Following equalization with CB, each blastomere formed a polar lobe at second cleavage, leading to two large and two small cells. Larvae from these embryos formed lobe-dependent structures: 75% with eyes, 92% statocysts, and 83% external shell. Duplication of at least one lobe-dependent structure was found in 67% of the larvae (50% with eye duplication). Duplication was scored as more than two eyes or statocysts or more than one foot or operculum. Following equalization with SDS, most blastomeres failed to form a polar lobe, resulting in four equal cells. The larvae formed fewer lobe-dependent structures: 61% eyes, 39% statocysts, and 17% external shell. Duplication was infrequent: 17% overall and 6% eye. The formation of lobe-dependent structures was improved when SDS-equalized blastomeres were separated at the 2-cell stage and raised in isolation: 85% eyes, 30% statocysts, and 30% external shell. Duplication occurred more frequently in larvae from such half embryos: 30% overall and 20% eye. Embryos from CD isolates can develop two eyes and two statocysts, while D isolates show some eye development, but reduced statocyst and external shell development. CB equalization may lead to CDCD embryos, while SDS equalization may lead to DDDD embryos. This study demonstrates that lobe-dependent structures often are duplicated in *Ilyanassa* larvae following equalization of first cleavage.

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Investigation of electroporation as a method to introduce exogenous materials into sea urchin eggs. CAROL SAAVEDRA (McGill University), RAYMOND T. KADO, AND BRUCE P. BRANDHORST.

Introduction of ions and molecules into cells has become useful in investigations of cellular physiology and gene expression. For instance, DNA has been microinjected into eggs and cells and is transcribed under appropriate gene regulatory conditions. We report here conditions for the electroporation of ions and molecules into eggs of the sea urchin *Lytechinus pictus* via the discharge of a capacitor charged with a variable power supply through a chamber (3.6 mm high, 29 mm long, 2.3 mm wide) having two platinum electrodes and containing 75 μl of an egg suspension (2000 eggs). A voltage discharge of 200 v, resulting in a current peak of 14 amps having an exponential time constant of 1.3 ms, allowed the entry of calf thymus DNA (1-10 kb, 3 mg/ml) prestained with Hoechst dye 33242 into zygotes from which the fertilization envelope had been removed; the dyed DNA concentrated in the pronuclei of up to 100% of the zygotes within 10 min, as visualized by epifluorescence microscopy. The medium (designed by Mazia to mimic the zygotic cytoplasm) consisted of 250 mM K-gluconate, 250 m*M* N-methylglucamine, 5 m*M* NaCl, 1 m*M* $MgCl_2$, 20 m*M* Hepes, pH 7.2. Lower voltages resulted in the entry of only smaller molecules: entry of calcium (resulting in activation of eggs in seawater) at 25 v, entry of inositol trisphosphate (MW 526, resulting in activation of eggs in calcium-free seawater) and carboxyfluorescein (MW 376) at 150 v, and entry of trypan blue (MW 960), FITC dextran (MW 150,000), and DNA at 200 v. Eggs could be activated by transfer to seawater (containing calcium) for up to 3 minutes after electroporation, indicating that membrane pores persist for a short period. A variety of media have been used for electroporation of eggs, zygotes, and embryos, but in no instance has cleavage occurred under conditions required for entry of calcium molecules into the egg.

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Amoeboid sea urchin eggs produced by cytochalasin B treatment have a disrupted actin staining pattern. ANNE-MARIE SCHOLER (Harvard University), ROY GOLSTEYN, HEIDE SCHATTEN, AND GERALD SCHATTEN.

Fertilized marine eggs can be induced to undergo an "ameboid" motion during cleavage. This motion has been induced in *Spisula* eggs by colchisine treatment, and in sea urchin eggs by hydrostatic pressure or cytochalasin B. The appearance is that of an unanchored cleavage furrow, or of a lobe propagating around the cell surface. This study investigated actin distribution in these eggs, to determine its possible involvement in this motion.

Lytichinus pictus eggs were fertilized in 10 micromolar para-aminobenzoic acid, and after 1.5 min transferred to 5 microgram per ml cytochalasin B in seawater for 10 min to prevent microvillar elongation. During this time, the fertilization envelopes were removed with a nytex screen. The eggs were washed and cultured in calcium-free seawater. Samples were removed at 10 min intervals from 45 to 85 min post-fertilization, and fixed for actin-specific rhodamine phalloidin staining or scanning electron microscopy. Detectable motion began between 45 and 60 min. Cleavage in the controls began after 55 min. Amoeboid motion continued for at least 2 h, unlike cell division. The cytochalasin B-treated eggs did not show microvilli in surface structure or in rhodamine staining as seen in controls. Also, the cortical actin network became increasingly disrupted with time, forming large gaps which corresponded with lobes or large blebs, and heavy bands which were associated with contractures of the cell. Small blebs also formed, which stained strongly for actin and were associated with the cortical bands of actin staining in contrast to the lobes. It is suspected that the loss of an intact cortical actin network is responsible for the large bleb formation. The cytochalasin B treatment prevents elongation of microvilli, as does hydrostatic pressure (Begg 1983, *J. Cell Biol.* **97**: 1795–1805). This may prevent the anchoring of the cortical actin for effective cleavage furrow formation. Combination of these two mechanisms could produce the observed movements.

This work was supported by NIH training grant 07098-010 to the Embryology course at the Marine Biological Laboratory, Woods Hole, Massachusetts 02543.

Cytoplasmic distribution of the M-phase-inducing protein cyclin A in early embryos. KATHERINE I. SWENSON (Harvard Medical School) AND JOAN RUDERMAN.

Fertilization of clam oocytes triggers the synthesis of a new set of proteins which are programmed by pools of maternally stored mRNA. Synthesis of at least a subset of these proteins is required for the newly fertilized embryo to complete meiosis or enter into mitosis of each successive cleavage division (Hunt and Ruderman, in prep). Two of these new proteins, cyclins A and B, have very unusual behaviors—they are synthesized continuously but are periodically destroyed at the time of the metaphase/anaphase transition of each mitotic cell cycle. These proteins may function in driving cleavage during cell divisions of early embryos.

Using an antibody generated against a cyclin A-bacterial fusion protein, we showed previously that cyclin A appeared to be absent in the oocyte and that it underwent the cell cycle-dependent accumulation and destruction not only during mitosis but meiosis as well. Cyclin A also induces M-phase when introduced into *Xenopus* oocytes (Swenson *et al.*, submitted).

Using the anti-cyclin A antibody for immunofluorescence staining we found that in cells prepared/fixed under a variety of conditions, this protein appeared to be a homogeneously distributed cytoplasmic protein at all cell cycle stages. There were differences in the relative degrees of staining for cells in different stages of the cell cycle. These differences were consistent with our previous labeling and immunoblot studies showing that the levels of cyclin A vary with the cell cycle.

The effects of UV irradiation on Ilyanassa obsoleta *early development.* MARK VELLECA (Yale University), KAREN SYMES, EVELYN HOULISTON, JOANN RENDER, AND RAY KADO.

UV irradiation of early embryos disrupts axis formation in several organisms, including amphibians, dipterans, and ascidians. We investigated the effects of locally applied UV light to *Ilyanassa* embryos. *Ilyanassa* embryos form a series of three polar lobes prior to first cleavage between which the embryos are spherical and naturally orientate vegetal pole downwards. Irradiation of vegetal material was achieved by suspending the embryos in a quartz cuvette 2.9 cm above a hand-held UV source (800 μW/cm^2, 253 nm) for 6 min.

An irradiated asynchronous population of rounded embryos with two polar bodies exhibited equalization of first cleavage in 21% of cases. Instead of normal trefoils, unipolar cleavage resulted in heart-shaped embryos; the presumptive lobe material is equally divided between blastomeres. Embryos were scored after 9 days, and 23% showed duplication of a lobe dependent structure: eyes. Synchronous cultures were irradiated following second polar lobe resorption and immediately prior to third polar lobe extrusion. Equalized cleavage occurred exclusively at the latter stage in 26% of cases. This culture was divided into heart and non-heart shaped embryos, duplication arising in 21% and 8% of cases, respectively. Embryos exposed following second polar lobe resorption, despite having normal trefoils at first cleavage, gave 20% duplication. In each experiment embryos lacking duplications exhibited a variety of morphologies.

These results imply that UV induced equalization of cleavage in *Ilyanassa* may influence the duplication of lobe-dependent characteristics. To explore this possible correlation further we designed a 9 μm fiber optic system that allowed irradiation of precise regions of the embryo. The power at the tip of the microbeam (120 μW/cm^2, 280 nm) was insufficient to cause any effect, but we feel that with a stronger source this will be a powerful tool for further work.

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Antibody-assisted studies of clam cyclin B. J. WESTENDORF AND J. RUDERMAN (Zoology Department, Duke University, Durham, North Carolina 27706).

Clam cyclin B protein is first synthesized from stored maternal message at fertilization and is made continuously thereafter. However, S-35 methionine labelling experiments indicate that cyclin B protein disappears at anaphase of meiosis II and mitosis 1 and 2. To investigate the behavior of cyclin B in the oocyte and during meiosis I and to localize it within the clam embryo, we cloned the message for cyclin B, fused the carboxy-terminal 80% of the coding region to bacterial TrpE protein, and made polyclonal antisera to the TrpE-cyclin B fusion protein. These antisera immunoprecipitated cyclin B protein from clam embryo lysates and recognized it on Western blots. The blots showed that cyclin B protein is present in the oocyte, does not disappear at anaphase of meiosis I, but does disappear at meiosis II and mitosis. When anti-cyclin B is bound to clam oocytes and localized with a secondary antibody conjugated to rhodamine, bright fluorescent spots are seen in the cytoplasm. Two to eight minutes after activation, some smaller fluorescent spots appear close to, on, or inside the nucleus. Ten min after activation the oocyte nuclear envelope breaks down, the fluorescent spots are small, numerous, and scattered throughout the cell. Thus, cyclin B has unusual dynamics during oocyte meiosis I; it relocalizes soon after oocyte activation and it does not disappear during anaphase I. This suggests that cyclin B function is different during the first reduction division than during other divisions or that cyclin B functions in events that are different in meiosis than in other cell divisions.

Voltage dependence of junctional conductance between Fundulus *blastomeres is altered by acidification.* R. L. WHITE, V. K. VERSELIS, D. C. BROSIUS, AND M. V. L. BENNETT (Marine Biological Laboratory).

Single blastomeres were mechanically isolated from 32–64 cell *Fundulus* embryos in culture medium (Leibovitz's L-15) containing <.05% colchicine to inhibit mitosis and then pushed together to form cell pairs. Each cell of a coupled pair was voltage clamped with independent circuits. In some cases, a double-barrelled microelectrode measured voltage and intracellular pH (pHi). Transjunctional voltage pulses (Vj's) of either sign decreased gj. Plots of steady-state Vj *versus* gj are fit by a Boltzmann relation with voltage sensitivity aproximately half that for amphibian blastomeres. A residual gj of 10–30% of the resting value remained at large Vj's. For applied Vj's greater than 20–30 mV gj decayed in two phases, fast and slow. The larger fraction (80–90%) decayed rapidly; at 50 mV the fast and slow time constants were 300–500 ms and 4–8 s, respectively. The slow decay was not affected by voltage while the fast time constant decreased with increasing Vj. Fluctuation analysis of junctional currents at Vj's where gj was at an intermediate value showed power density spectra. These were well fitted by a single Lorentzian. The corner frequency of 0.7 Hz corresponded to the fast time constant at the same Vj.

Normal pHi was 7.2 and bathing the cells with L-15 equilibrated with 100% CO_2 reduced gj and pHi concomitantly. Plots of pHi *versus* gj show steep titration curves with half-maximum gj's at about 6.5. Recovery was often complete, but displayed hysteresis in the alkaline direction of 0.15–0.3 pH units. Acidification blocked the voltage dependent component of gj; at pHi 6.4, the small residual gj was almost voltage insensitive. As pHi decreased further, gj was abolished.

We infer that there are at least two classes of gap junction channels between *Fundulus* blastomeres; the larger fraction has moderate voltage and pH sensitivity; the smaller fraction is less voltage and pH sensitive.

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Cellular events during the allorecognition reaction in the marine sponge, Microciona prolifera *(Ellis and Solander).* SVEN ZEA, SUSAN MOCHEL, AND TOM HUMPHREYS (Cancer Research Center, University of Hawaii).

Apposed tissues from different individuals of *Microciona prolifera* exhibited an allogeneic reaction in all 71 combinations tested. The two pieces of tissue adhere upon contact and initiate the reaction which becomes readily visible in the contact zone by 3–6 h as a line of yellow due to the accumulation of an inmigrating, multigranular, wandering cell type, called the gray cell. These cells appear gray under transillumination and their cytoplasmic granules autofluoresce yellow under UV epifluorescence. The inmigrating cells line up with archeocytes and by 2–4 days an extracellular barrier 5 to 10 μm thick is produced between the tissues. No cytotoxic reactions are evident during any part of this reaction which appears to represent the sponge's general response to foreign organisms.

Small suspended aggregates made from cells of one or of another individual usually show allogeneic reactions when they are brought together. The ability of small groups of cells to generate this rapid allogeneic reaction suggests that it is triggered by the lack of a "self" signal between the cells of two individuals rather than by a positive recognition of "foreign" epitopes by differentiated cells with a diversity of specific receptors. This impression is supported by experiments in which killed tissue, either frozen in liquid nitrogen or heated to 56°C or 80°C, elicited the allogeneic reaction from live tissue of the same individual. However, the reaction must include recognition of "living" tissue since the sponge does not react to wood, plastic, or glass. Sponge allorecognition appears to be a two-signal system with an inhibitory "self" signal and a positive, relatively nonspecific "living tissues" signal which, in the absence of the former, triggers the release of lymphokin-like, chemotatic molecule(s) that stimulate and organize the cellular events.

Supported by NSF.

ECOLOGY

A comparison of predation on post-larval American lobsters by cunners and mud crabs on three different substrates: eelgrass, mud, and rocks. D. E. BARSHAW AND K. L. LAVALLI (Boston University Marine Program, Marine Biological Laboratory).

Groups of ten late stage IV or early stage V lobsters were introduced into seawater table sections consisting of either a bare mud substrate, a rocky substrate (with some rocks covered by *Fucus* spp.), or an eelgrass substrate, all collected from subtidal areas. The lobsters were given four days in which to build burrows. Predators were then introduced into all sections, except those which had been designated as predator-free control tanks. These latter tanks served as an estimate of the natural mortality of the lobsters within each substrate type without predation. After four more days the predators were removed and the number of surviving lobsters was counted and compared to the number surviving in the predator-free control tanks. Censuses were taken four times daily during the eight days of the experiment to determine how many lobsters were without burrows. We also observed the behavior of the predators and lobsters in each of the experimental sections for a one-half hour period after the introduction of the predators.

Establishment of burrows occurred most rapidly in the rock substrates, followed by the eelgrass, with the lobsters in the mud taking longest. No difference in survival was found in any of the predator-free control substrates. When subject to predation by non-burrowing fish (the cunner, *Tautogolabrus adspersus*), more lobsters survived in the rocky substrates than in either the mud substrates or the eelgrass (Chi-square, $P < 0.001$). Furthermore, significant differences were found between the mud controls and experimentals, and the eelgrass controls and experimentals (Chi-square, $P < 0.001$); however, no significant differences were found between the rock controls and experimentals. Thus predation occurred in both the mud and eelgrass substrates with the fish as predators. No predation occurred in the rock substrates, indicating that lobsters burrowed under rocks are protected from non-burrowing predators. When subject to predation by burrowing mud crabs (*Neopanope texani*), a significant difference occurred between the experimentals and controls in the mud substrates (Chi-square, $P < 0.001$). Therefore, lobsters burrowed in mud substrates are not protected from predation by small, burrowing crabs. Experiments are still in progress on the eelgrass and rock substrates using mud crabs as predators.

We thank Donald R. Bryant for his help in collecting substrates. This study was supported in part by a NWF Environmental Conservation Fellowship.

Optimal foraging with nutrient constraints and patchy distributions. JOHN F. BOYER (Union College).

Computer simulation was used to evaluate alternative feeding strategies for a predator which must obtain a minimum quantity of three different nutrients. The three prey species differ in their nutrient composition and abundance, and also may be aggregated within patches. The domain within the parameter space for which an "eat-everything" strategy is superior to a "minimal-consumption" strategy was determined. Aggregation within patches increased both the mean and variance of total costs (measured in arbitrary time units) for all strategies. If search costs were greater than 20% of consumption costs, the eat-everything strategy was almost always superior. Emigration with traverse times of the order of 2× to 10× search times was advantageous only for the greatest extremes of nutrient disparity and abundance (ratios between 20:10:1 and 30:1:1). The decision rule for emigration was based on acquiring a minimum of x nutrient units in the n most recent prey encounters—a wide range of values (from 1 to 10 for n, and 2 to 16 for x) lead to similar total costs (consumption plus search plus traverse times). Therefore two simple and robust decision rules suffice for a "nearly optimal" predator.

This work was supported in part by a grant from Union College, Schenectady, New York.

Control of phytoplankton growth in a brackish coastal pond. FRANCISCO A. COMIN (Department of Ecology, University of Barcelona, Spain).

A batch culture experiment combining salinity and nutrient enrichments ($160\ \mu mol\ l^{-1}$ of nitrogen as NH_4^+ or NO_3^-, $10\ \mu mol\ l^{-1}$ of phosphorus as phosphate) was performed in continuously shaken and aerated conditions at constant temperature and light. The purpose of the experiment was to identify the specific nutrients limiting the growth of phytoplankton from Oyster Pond (O.P.), Falmouth, Massachusetts (salinity 4 ppt, $1.5\ \mu mol\ N\text{-}NH_4^+\ l^{-1}$, $0.35\ \mu mol\ N\text{-}NO_3^-\ l^{-1}$, $0.42\ \mu mol\ P\text{-}PO_4^{3-}\ l^{-1}$). Phytoplankton biomass was measured as *"in vivo"* fluorescence. Species composition was studied using an inverted microscope.

The peaks of total phytoplankton biomass were 10–40 times and 10–30 times greater in the $NO_3^- + PO_4^{3-}$ and in the $NH_4^+ + PO_4^{3-}$ treatments, respectively, than in the controls in the four salinity environments performed (O.P. water, O.P. water plus $14\ g\ NaCl\ l^{-1}$, O.P. water plus $12\ g\ NaCl\ l^{-1}$ plus $2\ g\ Na_2SO_4\ l^{-1}$, phytoplankton from O.P. in filtered Sargasso Sea water). No significant increase was observed in the other treatments. Both nitrogen and phosphorus are thus key limiting nutrients for growth of phytoplankton.

We also recorded the response of blue-green algae to each of the experimental treatments. No *Anabaena* filaments were observed in the control Sargasso Sea jars on six days of culturing. No significant change in the blue green abundance took place in O.P. water without addition of nutrients. A greater decrease occurred in the unenriched jars with sulfate than in those where only sodium chloride was added.

The number of *Anabaena* filaments increased considerably in the nitrogen plus phosphorus enriched water from O.P. No clear changes were observed in the same nutrient enriched water where sodium chloride alone was added. A decrease occurred in those nitrogen plus phosphorus enriched jars where sulfate was added, higher in the enrichments with nitrate plus phosphate than in the ammoniun plus phospate one.

The results thus show a higher inhibitory effect of the sulfate than the sodium chloride on the *Anabaena* population growth. Further, the potential response to nitrogen addition by blue greens is prevented by the presence of sulfate.

Thanks are given to BUMP, I. Valiela, A. Chatterjee, D. Kulis, and D. Stoecker for help in this study.

Inflow and outflow of phytoplankton at Great Sippewissett salt marsh: a twenty-four hour survey. GEORGES DIOGÈNE (University of South Alabama).

A 24-hour survey at Great Sippewissett salt marsh in August, 1986, covered two tidal cycles. The station was at the mouth of the channel that connects the marsh with the sea. Water samples were taken every hour, filtered, and fixed. Temperature and salinity were recorded. Water flow was calculated from measurements of current speed and tidal height. Aliquots of the samples were placed in sedimentation chambers in the laboratory and cells were counted using an inverted microscope.

Diatoms, which usually dominate turbulent waters, composed most of the phytoplankton. Few dinoflagellates and Cyanophyceae were present. Diatoms were divided into three groups: *Chaetoceros* spp. (average 85% of the total) other planktonic species, and benthic diatoms.

Cells per liter reached a maximum at high tide and a minimum at low tide. (Averages 260,000 cells/l in flooding tide and 94,000 cells/l in ebbing tide.) Planktonic species including *Chaetoceros* had a maximum at high tide and a minimum at low tide. Benthic diatoms had their maximum at low tide and their minimum just after high tide. The percentage of planktonic diatoms varied significantly during tidal cycle and comprised 95% during flooding tide and 91.5% during ebbing tide. The outflow of planktonic diatoms was 43% higher than the inflow whereas the outflow of benthic diatoms was 187% higher than the inflow. Thus there was a possible loss of benthic diatoms from the marsh sediment and consumption of planktonic cells in the marsh.

Total cells were calculated for flooding and ebbing tides. The amount of cells entering the marsh during the flood tide was 16×10^{12}, whereas the amount of cells leaving was 24×10^{12}, resulting in a net outflow of cells from the marsh. The outflow of water was greater than the inflow of water and may account for this loss.

I thank people who helped me in my project and the MBL for the financial aid.

The role of sulphide in the settlement of Capitella *sp I larvae.* NICOLE DUBILIER (University of Hamburg, Federal Republic of Germany).

The behavior and settlement time of *Capitella* sp I larvae was observed in the presence of H_2S. Individual 55 mm diameter glass petri dishes were filled with 30 ml of 0.2 μm filtered deoxygenated seawater and the amount of Na_2S stock solution needed to reach a H_2S concentration of 1.0 m*M* was added. This concentration is optimal for the settlement of *Capitella* sp I larvae (Cuomo 1985, *Biogeochemistry* **1**: 169–181) and corresponds to concentrations found in H_2S rich sediments. The pH was adjusted to 7.9, and temperature (21–22°C) and H_2S concentrations recorded for each experiment. In each parallel experiment five freshly hatched larvae were pipetted into a dish and covered with a watch glass; only larvae from the same brood were used in parallel runs.

In experiments without sediment, larvae never settled in the controls (0 m*M* H_2S) even after 2 to 3 days (n = 10). In the dishes with H_2S, 50% settled after 4 ¼ hours, 100% after 8 hours (n = 10).

In experiments with a small clump of azoic, organic-rich mud in the middle of the dish, 85% of the larvae settled in this mud within 5 min at 0 m*M* H_2S, and 98% within 15 min. Of 120 larvae tested, only 2 needed >15 min to settle. In 1.0 mM H_2S, only 43% settled within the first 15 min; it took up to 4 hours for all larvae to settle (n = 50). The percentage of larvae that settled within the first 15 min at 1.0 mM H_2S varied (0–80%) within individuals of one brood and between broods.

H_2S clearly is not the only settlement cue for *Capitella* sp I larvae. Organic mud elicits settlement within minutes whereas the settlement response to H_2S is over a time range of hours.

Experimental studies of the feeding ecology of Scoloplos *spp. (Orbiniidae: Polychaeta) from Barnstable Harbor and Boston Harbor.* KATHLEEN FALK (University of Massachusetts, Boston).

Experimental microcosms were used to study surface biodeposition rates of organic carbon and total sediment resulting from the feeding activity of *Scoloplos* spp. In one experiment, worms collected from

Barnstable and Boston Harbors were cultured in their native sediments. In a second experiment worms were cross-cultured between sediment types. Barnstable sediments were characterized as sandy (63% greater than 250 μm), with low organic carbon (0.68%), whereas Boston Harbor sediments were primarily fine (9[illegible] less than 250 μm), with a higher organic carbon content (3.14%).

Biodeposition rates per unit biomass of worms cultured in their native sediments were 17.9 and 8.9 mg dry weight of sediment per day for Barnstable and Boston Harbors, respectively. Average percent organic carbon in Barnstable biodeposits was 1.07%, and in Boston Harbor biodeposits, 2.8%. Calculated assimilation efficiencies were 36.9% for Barnstable worms (gross growth efficiency 9.5%), and 20.6% for Boston Harbor worms (gross growth efficiency 6.7%).

In cross-culture experiments, biodeposition rates of organic carbon and total sediment were similar within but different between sediment types. Biodeposition rates, assimilation efficiencies, and growth rates were higher in each case for worms grown in Barnstable *versus* Boston Harbor sediments. Boston Harbor worms showed weight-specific growth rates of 4.0% per day in native and 10.3% per day in Barnstable sediments. Weight-specific growth rates of Barnstable worms were 7.5% per day in native *versus* 1.0% per day in Boston Harbor sediments. Results are interpreted in support of optimal foraging theory.

Grazing effects of Littorina littorea *in different habitats.* IRIS D. FREY (Philips University, Marburg, Federal Republic of Germany).

Littorina littorea specimens were collected at three different sites near Woods Hole, Massachusetts: a saltmarsh and two different rocky shores. Snails were caged at different densities. The cages were equipped with glass slides for the settlement of fouling organisms and placed in Eel Pond. Other cages were filled with rocks for algae settlement and placed at northwest gutter, Naushon Island.

Periwinkles fed on settling and already-settled fouling organisms. Grazing was selective in all cases: only the bryozoan *Bugula* ssp. and the tunicate *Botrylloides* were avoided. Grazing was a function of grazer numbers. There was no difference in effect between littorines from different sites. No habituation to their food sources was seen.

The grazing effect of algae (estimated from chlorophyll *a* per cm^2 rock-surface in cages) also was a function of *L. littorea* density. Naturally occurring densities are high enough to reduce algal growth drastically. There was no difference in growth between free-living, marked snails and snails in cages.

Replacement and displacement experiments with periwinkles from Naushon Island and Nobska Point showed no difference due to place of origin. Growth was higher at the Nobska Point site. At Naushon Island growth rate depended on the length of the experiment. The shorter it ran the higher was the growth rate.

I thank the MBL and the 'Studienstiftung des deutschen Volkes' greatly for their financial support.

The effects of fuel oil on mortality and feeding of the snail Melampus bidentatus. PAULA GRONDA (Instituto Nacional de Chagas, Argentina).

The effects of number 2 fuel oil on the snail *Melampus bidentatus* were examined in petri dishes containing three different substrates (detritus, cyanobacteria mats, and mud). Measures of feeding related activities included numbers of fecal pellets produced and holes dug into the sediment in thirty-six hours. Mortality was recorded every twelve hours for a seven-day period. Fuel oil (conc: 0.1, 0.3, 0.8, 2.0 mg of fuel oil/g wet sediment), were added to petri dishes except for the control dishes. Ten adult snails (average length: 7 mm) were placed in each dish. The treatments had six replicates.

The number of fecal pellets released and of holes produced decreased significantly as the fuel oil concentration increased for all substrates. Thus, fuel oil may be modifying *M. bidentatus* feeding activities.

Mortality was highest in dishes with contaminated cyanobacterial mats and lowest with contaminated mud. A fuel oil film was observed only on the surface of the mats. The cyanobacterial mats may not adsorb oil as contrasted with the other two substrates.

I acknowledge Ivan Valiela for directing my project, and Judy Capuzzo and Donald Rice for their help.

A device to measure intertidal wave force in channels. ROBERT HART (University of California, Berkeley, Department of Biophysics).

Narrow channels (<10 cm) between large rocks in the intertidal create a microenvironment of higher than ambient wave action. This is thought to occur because these channels narrow the water flow thereby accelerating it. A device described by Denny (1983, *Limnol. Oceanogr.* **28:** 1269–1274) was modified to measure wave force on organisms in this microenvironment. The device consists of a lever arm approximately 25 cm in length suspended within a Plexiglas box mounted over the channel. The lever arm descends up to 20 cm into the channel. An organism (or suitable model) is attached to the end of the lever arm. The impact of the wave thus moves the lever arm a distance which is recorded on a smoked slide within the box. The magnitude of the force is determined in the lab by suspending known weights from the arm, and moving them out the arm until the recorded deflection is matched. Assuming that this force acts only on the organism, a velocity is calculated from which the drag force of the moment arm is estimated. This is subtracted from the initially measured force, and an iteration routine followed to obtain the actual force on the organism. Initial measurements were made with the device at Nobska Point Lighthouse, Woods Hole, during 11–17 August, 1986, over three tidal cycles. An average value of .52 N for the snails *Littorina littorea* and *Urosalpinx cinerea* was obtained.

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Effect of segment loss on reproductive output in Capitella *species II (Polychaeta).*

S. D. HILL (Michigan State University), J. P. GRASSLE, AND J. T. BOYLAN.

The ability of polychaetes to replace lost segments is well-documented; however, the effect of segment loss and subsequent regeneration on reproductive output has received little attention. Because reproduction and regeneration are both major energy-requiring processes, the response of animals to segment loss could be an important factor in determining reproductive success. *Capitella* species II is an opportunistic species occurring in disturbed areas in the vicinity of Woods Hole, Massachusetts. In field samples, up to 100% of mature animals collected may have regenerating posterior segments. We have investigated the effect of segment loss on reproductive output.

Posterior segments were amputated every 4 weeks from a group of 62 female worms that were within 3 weeks of each other in age. Amputations were made at approximately the 25th abdominal segment, a level just posterior to the egg-producing segments. A control group of 67 females was selected from the same cohort and matched with respect to female parentage, size, and stage of vitellogenesis. Animals were paired with males and maintained in sieved, azoic mud at 20°C. Numbers of developing embryos in successive brood tubes produced by individual females were counted. By the seventh week females undergoing simultaneous regeneration and reproduction produced fewer eggs than animals undergoing only reproduction. Number of eggs per female, number of eggs per brood, and number of brood tubes produced were significantly reduced in the regenerating group.

In a reciprocal experiment, a group of juveniles from the same cohort which developed into males, and so did not undergo vitellogenesis, regenerated significantly more segments in four weeks following caudal amputation than did the reproducing females. It appears then that extensive segment loss in females of *Capitella* species II results in competition for resources and that both processes—reproduction and regeneration—may be compromised if the two occur simultaneously.

Support for this research was provided by NSF-ROW, OCE-8509169 (S.D.H.).

Effects of submergence and exposure on meiofaunal colonization and residency on Diopatra cuprea *(Polychaeta: Onuphidae) tube-caps.*

JOHN M. KASMER (University of Vermont).

Laboratory microcosms were used to assess whether different sized organisms of different broad taxonomic groups use the tube-caps of the polychaete worm *Diopatra cuprea* as a temporary or permanent habitat.

Tube-caps and sediments were collected from a sandy intertidal area of Barnstable Harbor, Massachusetts. Some were defaunated by twice freezing and thawing in the laboratory. Each of the following combinations of sediments and tube-caps were placed in four 400 ml glass beakers: defaunated sediments + natural tube-caps; natural sediments + defaunated tube-caps; and for a control, defaunated sediments + defaunated tube-caps. The beakers were filled with filtered seawater and aerated for 10 hours. Half the beakers were then drained and the experiment was continued for another 10 hours. The fauna on the tube-caps and in sediment samples were separated into three size fractions and counted. The fractions of animals occurring on tube-caps was then calculated. Analyses of variance were performed on data for the most abundant groups: nematodes and copepods. Providing only one source of meiofauna in each beaker permitted testing whether the epibenthos remained on tube-caps when they were exposed, and whether tube-caps were suitable or preferred habitats during both exposure and submergence.

For nematodes, there were significant differences between all sediment + tube-cap combinations, but not between exposure treatments or size classes. This supports the hypothesis that as a functional group, nematodes are residents on tube-caps, at least over a 20-hour period. The only significant term for copepods was an interaction, suggesting inconsistent behavior, and therefore suggesting transience on tube-caps. Annelid worms, present in lower numbers, were residents on tube-caps.

I thank the MBL for the financial support that allowed me to do this research, and the instructors of the Marine Ecology Course for their help.

The ability of post-larval American lobsters to remove plankton from the water column. K. L. LAVALLI AND D. E. BARSHAW (Boston University Marine Program, Marine Biological Laboratory).

Twenty late stage IV or early stage V lobsters were placed in 4–5 cm long pieces of glass tubing (diameter 1 cm) screened on both ends to prevent their escape. These tubes were then placed into jars containing 750 ml of filtered, aerated seawater. After a 24 h starvation period within these tubes, a 10 ml sample of sieved fluorescently dyed plankton was introduced into the ten experimental jars while an identical volume of dyed, filtered seawater (without plankton) was placed into the ten control jars. The lobsters were allowed 60–90 minutes to draw the plankton into their tubes, catch, and consume them. Following the feeding period, one investigator removed the lobsters from the tubes and the other investigator scored the lobsters. At no time did the investigator scoring the lobsters know whether the animal was a control or an experimental. Scoring was accomplished by placing the lobster (which at stage IV or early stage V is transparent) under an ultraviolet light in total darkness and noting whether dye was present in its digestive tract. Three size ranges of plankton were tested: 500 μm–1 mm, 250 μm–500 μm, and 125 μm–250 μm, and in all cases a significant number of lobsters were found to feed on the plankton (Chi-square, $P < 0.001$). No controls were ever scored as containing dye in their stomachs or intestines. The vitally dyed plankton typically consisted of adult and juvenile copepods, invertebrate larvae, and algae.

These results clearly indicate that lobsters are capable of removing swimming plankton from the water column. Observations showed that once the lobsters alerted to the plankton, they typically began pleopod fanning, thus drawing water containing plankton under their mouthparts and body. Scanning EM investigations on sequential changes in the mouthparts indicate that the numbers and types of setae increase as the lobsters pass from larval stages to post-larvae (Factor 1978, *Biol. Bull.* **154:** 383–408). While no measurements of setal spacing have been taken, the numbers and types found on the mouthparts may allow lobsters to suspension feed on small particles. Whether lobsters capture small parcels of water containing food particles with their mouthparts (as seen in calanoid copepods) or feed raptorially on the particles drawn into the tubes (which simulated their burrows) is unknown at this time and is under investigation with high speed cinematography. These investigations are continuing on the lower size ranges of 100 μm and 70 μm with fluorescently dyed beads.

This study was supported by a grant from the Lerner Gray Fund for Marine Research of the American Museum of Natural History.

Cyanobacterial diseases of the horseshoe crab (Limulus polyphemus). LOUIS LEIBOVITZ[1] (Laboratory for Marine Animal Health, Marine Biological Laboratory).

Diseases of horseshoe crabs (*Limulus polyphemus*) are reviewed. Previously unreported cyanobacterial diseases observed during a five-year period (1981–1986) in the Woods Hole area, are reported. Two principal clinical types of disease were noted, an acute and a chronic form.

The acute, highly fatal form of the disease occurred during spring and summer. A heavy, grossly visible, growth of filamentous cyanobacteria (blue-green algae), principally *Oscillatoria* spp., was found on the external gill lamellar surfaces of *Limulus*. The joints of the appendages and the ventral vascular sinuses were swollen. The more elastic ventral portions of the body and gills expanded ventro-laterally. Gill lamellae were ballooned outward. With extreme body swelling, the ventral body wall and gills ruptured, resulting in hemorrhaging and explosive release of large foul-smelling tissue fragments. Mortalities ranged from approximately 30 to 70 percent in captive populations.

Histologically, the acute disease was characterized by penetration of the trichomes into the lamellar walls, dilation of the lamellar sinuses, vasculitis, and presence of trichomes in the blood. Other bacterial septicemias frequently followed the above lesions.

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The chronic form of the disease occurred throughout the year. Heavy growth of small unicellular colonial types of cyanobacteria (order Chroococcales) were found on the gill lamellar surfaces. The principal post-mortem sign, grossly, was blackened gills. Mortalities ranged from approximately less than one, to ten percent. The disease could be diagnosed best by microscopic examinations of the gill lamellae. The organisms formed an extensive laminated golden-colored mosaic layer which did not penetrate but covered and occluded the respiratory surfaces. As the disease progressed, the developing organisms became blackened.

This study is supported in part by a grant from the Division of Research Resources, National Institutes of Health (P40-RR1333-06).

Growth and chemical composition of Ulva *cf.* rigida *C. AG. cultured in different seawaters at various levels of light and nitrogen.* CARLO MORUCCI AND ANNAMARIA SPANÓ (Dipartimento di Biologia Vegetale, Universitá di Roma "La Sapienza").

The effect of nutrients, light, and waters of different origin on the growth of *Ulva cf. rigida* C. AG. was investigated. *Ulva* was collected from Green Pond and kept in low-nutrient water for 36 h. Water collected from Perch Pond, Vineyard Sound, and the Sargasso Sea was filtered at 0.2 μ and diluted to the same salinity (25 ppt.). The initial content of NH_4^+, $PO_4^=$, NO_3^-, and NO_2^- in that water was determined.

NH_4^+ was added to cultures at levels one, two, and three times that of its initial concentration in Perch Pond (*i.e.*, 7.6, 15.2, 22.9, and 30.5 μM). *Ulva* discs, cut from the central, expanded region of the mature thallus, were placed in 800 ml jars each containing a different NH_4^+ concentration. The discs were incubated in the jars at 20 ± 0.5°C for 48 h.

Three different light levels were obtained by screening the jars. The light of a cool solar lamp was 100 $\mu E\ m^{-2}\ s^{-1}$ and the three intensities used for *Ulva* were 43, 21.5, and 10.2 $\mu E\ m^{-2}\ s^{-1}$. Increased light yielded only minor differences in growth rate and only at high concentrations of NH_4^+.

Algae growth was estimated by measuring the disc area with a digitizer. A linear increase of the specific growth rate with increased NH_4^+ demonstrated that nutrients was the most important factor for growth under these light conditions.

There were also significant growth differences in seawaters from the Sargasso Sea, Vineyard Sound, and Perch Pond. These differences were not accounted for by macronutrients (NO_3^-, NO_2^-, $PO_4^=$) whose uptake was determined.

Na, K, Mg, and Ca analysis of the algal tissue gave no significant differences. Mn content was higher in the high NH_4^+ treatments. Fe was lower in *Ulva* from Sargasso Sea and Perch Pond than from Vineyard Sound.

Thus, *Ulva* seems suitable for bioassays of different waters.

We thank Prof. L. Provasoli for his help with portions of this work, the staff of the Marine Ecology course, and the MBL for financial aid.

Feeding versus *space competition in the colonial tunicate* Botrylloides *as seen on fouling plates in Eel Pond, Woods Hole, Massachusetts.* PHILIP E. MYERS (University of South Carolina).

The majority of studies on competition in fouling communities have examined space competition. I examined whether competition for food is also important within these communities.

Fouling plates were placed in Eel Pond for four days, after which they were brought into the laboratory, manipulated, and returned to the field. Two colonies of one to three zooids were choosen for study on each plate. Every other day from 29 July to 16 August 1986, the number of zooids in each colony was counted.

Three treatments are reported here. In the first treatment everything was removed from the plate except two isolated *Botrylloides* colonies. This eliminated all competitors for both space and food. In the second treatment (control), two colonies were selected for study but nothing was removed. In this treatment space and possible food competition occurred. In the third treatment two *Botrylloides* colonies were picked and everything was removed that was within 2 mm of each colony. In this treatment space competition was eliminated but many animals still surrounded the colonies and competition for food could occur.

Growth was exponential and the rate for each colony was obtained by regression. Mean growth rates were examined using an analysis of variance. Significant differences were found between all three treatments with *Botrylloides* growing fastest when isolated and slowest in the control treatment. This experiment shows that competition for food did occur because both the feeding and isolated treatments did not have competition for space and the isolated treatment grew at the fastest rate.

I thank the Marine Ecology course at MBL for making this study possible.

Seasonal changes in plant growth rate and root glutamine synthetase along a natural depth gradient in an eelgrass (Zostera marina) *meadow.* A. MARSHALL PREGNALL (Vassar College).

Eelgrass (*Zostera marina* L.), a perennial submerged angiosperm, occurs between 1 and 6 m depth in Great Harbor, Woods Hole, Massachusetts. In summer, plant size increases with growing depth, while plant abundance (#/m^2) decreases with depth. Plant relative growth rates (RGR) decline to 5–10 $mg \cdot g^{-1}$ day^{-1} during winter and are equivalent along the depth gradient. Growth accelerates in late spring, when plant RGR increases with depth. Flowering shoots grow more rapidly than vegetative shoots. Growth is maximal in June, when plants throughout the meadow have equivalent RGR (30 $mg \cdot g^{-1}$ day^{-1}). Plant biomass is maximal during late summer, while growth is declining (17–22 $mg \cdot g^{-1}$ day^{-1}).

Plant nitrogen assimilation potential was assessed during the year by measuring root glutamine synthetase (GS) activity with the synthetase assay at 35°C (1 unit = 1 $\mu mole \cdot g^{-1}$ fresh weight $hour^{-1}$). In August, GS activity increased 5-fold down the depth gradient (15 units shallow to 75 units deep) and was more than sufficient to support growth nitrogen demand. Deep-growing plants have lower activity from December to May (20–30 units) than during summer (60–80 units). Mid-depth plant GS activity declines from 40 units in fall to 20 units in May and increases again through summer. Shallow-growing plants have low activity in August (15 units), higher activity in winter and spring (20–40 units), and decreasing activity through summer.

GS activity was also measured at ambient water temperature (maximum 22°C in August, minimum 1°C in March). Activity was low for all depths from December to March (<3 units). Increasing water temperature in late spring through summer permits greater GS activity. During summer, deep-growing plants had highest activity (30–35 units), mid-depth plants had intermediate activity (18–25 units), and shallow-growing plants had low activity (7–10 units).

Research was supported in part by University of Massachusetts-Five College participation in the Woods Hole Marine Sciences Consortium.

Glutamine synthetase activity in roots of salt marsh plants: effects of species, height form, and nitrogen availability. CAROL S. RIETSMA (State University of New York, New Paltz) AND A. MARSHALL PREGNALL.

Long-term nutrient enrichment of experimental plots at Great Sippewissett salt marsh near Falmouth, Massachusetts, has resulted in vegetational changes including (1) replacement of *Spartina patens* and *S. alterniflora* by another grass *Distichlis spicata* (Valiela *et al.* 1985, Pp. 301–315 in *Ecological Considerations in Wetlands Treatment of Municipal Wastewaters,* Van Nostrand Reinhold) and (2) a change from short to tall form of *S. alterniflora* (Valiela *et al.* 1978, *Am. Nat.* **112:** 461–470). We hypothesize that differential capacity of plant species to assimilate ammonium is responsible for these changes.

To test this hypothesis, we determined the activity of the enzyme glutamine synthetase (GS) which catalyzes ammonium assimilation. GS activity was detected at 35°C by the transferase assay (O'Neal and Joy 1974, *Plant Physiol.* **54:** 773–779) in root extracts of *D. spicata, S. patens,* and short and tall *S. alterniflora* collected from enriched and control marsh plots.

GS activity in *D. spicata* was 180 ± 14 (mean ± S.E.) $\mu moles\ g^{-1}$ fresh wt h^{-1}. Activities in *S. patens,* short *S. alterniflora,* and tall *S. alterniflora* were lower, averaging 75 ± 5, 56 ± 13, and 79 ± 17 $\mu moles\ g^{-1}$ fresh wt h^{-1}, respectively. In all species activity increased slightly but not significantly with fertilization.

Distichlis spicata may replace *S. patens* and *S. alterniflora* in fertilized plots because it has higher capacity to assimilate ammonium. In control plots edaphic conditions may prevent *D. spicata* from attaining its high potential assimilatory capacity and replacing other species. The change from short to tall *S. alterniflora* in enriched plots cannot be attributed to assimilatory capacity because enrichment did not increase significantly the assimilatory capacity of short plants. Changes in edaphic conditions with enrichment may be responsible for the height change.

Supported by a SUNY New Paltz Grant for Research and Creative Projects to Carol S. Rietsma.

The effect of colony size in intraspecific competition for space in the hydroid Hydractinia echinata *(Flemming).* FERNANDO A. ZAPATA (Department of Ecology and Evolutionary Biology, University of Arizona).

To test the hypothesis that colony size affects the outcome of intraspecific competitive interactions in the athecate colonial hydroid *Hydractinia echinata,* two stoloniferous clones were grown on microscope slides in pairwise combinations of two sizes in a two-way factorial design.

Cultures of the two genotypes used in this experiment were made according to L. Buss *et al.* (1984, *Biol. Bull.* **167:** 139–158). In each slide, two colonies of different genotype were grown in one of four combinations of size and genotype: large 1 × large 2; large 1 × small 2; small 1 × large 2; and small 1 × small 2. Each treatment was replicated five-fold for a total of 40 colonies growing on 20 slides.

The growth rate of each colony was calculated from 4 measurements over 12 days by fitting an exponential curve to the increments in the area of the colony mat. Differences in growth rate between the two genotypes and among the four treatments were analyzed with a two-way ANOVA. There was a significant difference between the growth rates of the two genotypes ($F_{1,32} = 4.42$; $P < 0.05$), but there were no significant treatment effects and no significant genotype × treatment interaction.

The lack of treatment effects may be explained by the short duration of the experiment. Stolon-stolon and stolon-mat interactions occurred between colonies but there was not sufficient time for mat-mat contact in any of the 20 slides. A longer-term study is required to determine the effect of colony size in intraspecific competition in this hydroid.

Leo Buss provided advice and materials. I thank Rick Osman, Peter Frank, and the MBL Marine Ecology course staff. Financial support was provided by a Lucille P. Markey Charitable Trust scholarship and a Lucretia Crocker scholarship.

FERTILIZATION

Fertilization potential and polyspermy prevention in the egg of the hydrozoan Hydractinia echinata. CELESTE BERG, COLLEEN KIRBY, DOUGLAS KLINE, AND LAURINDA A. JAFFE (Department of Physiology, University of Connecticut Health Center, Farmington, Connecticut).

To examine whether a fertilization potential and electrical polyspermy block occur in hydrozoans, we recorded the membrane potential during fertilization from eggs of *Hydractinia echinata.* Eggs and sperm were obtained by putting isolated gonophores in the dark for several hours, then transferring them to light to induce spawning (Ballard 1942, *Biol. Bull.* **82:** 329–339). The resting potential of the unfertilized egg was −72 ± 3 mV (S.D., n = 13). One to two minutes after the addition of sperm to the recording dish, the egg membrane depolarized slightly; the peak of the response was −64 ± 4 mV (n = 13), and the potential returned to its original level after 5 ± 1 min (n = 13). Recordings were continued through first cleavage, which was normal in all of the embryos. During this time no further change in membrane potential was observed. By contrast, eggs of various higher invertebrates (nemerteans and many protostomes and deuterostomes) produce fertilization potentials with an amplitude of about 100 mV; these fertilization potentials function in the prevention of polyspermy (see Kline *et al.* 1985, *J. Exp. Zool.* **236:** 45–52). However, in *Hydractinia,* the fertilization potential does not serve to block polyspermy, since eggs inseminated during voltage clamp at −65 to −50 mV (similar to the peak attained during the fertilization potential) were fertilized and cleaved normally (n = 4). The mechanisms for polyspermy prevention in hydrozoans are unknown, but polyspermy can be induced by raising the external sperm concentration: polyspermy occurred in 26 to 83% of the eggs (3 experiments) when the sperm concentration was increased ten times over a concentration which produced monospermy in all eggs. This observation argues against the possibility of a polyspermy block resulting from the restriction of sperm entry to an area so small that only one sperm can enter. In conclusion, neither an electrical block nor the restriction of sperm entry to a single site on the egg surface can account for polyspermy prevention in *Hydractinia.*

This work was supported by an NIH training grant (5-T35-HD07098) awarded to the Embryology Course, Marine Biological Laboratory, Woods Hole, Massachusetts, and by NIH grant HD 14939 to L.A.J.

Activation of surf clam (Spisula solidissima) *oocytes by phorbol ester.* FRANÇOIS DUBÉ (University of Quebec at Rimouski) AND ROY GOLSTEYN.

Increased Ca^{2+} influx is the primary trigger that reinitiates maturation (germinal vesicle stages) in protostome oocytes arrested at prophase I of maturation. The possible involvement of protein kinase C in this process was investigated with *Spisula* oocytes by testing the effects of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA induces germinal vesicle breakdown (GVBD) with an ED_{50} of 18 n*M* (see also Eckberg and Szutz, this issue). GVBD is slow to occur (40–90 min), asynchronous, and is followed by first polar body extrusion and subsequent formation of a variable number of pronuclei. These effects are not observed after addition of the less potent methylated derivative of TPA, TPA-Met. Other effects of TPA include: (1) activation of an Na^+-dependent acid release quantitatively similar to that induced by K^+-activated or fertilized oocytes but which shows slower kinetics; (2) a change in protein synthesis pattern characterized by the increased synthesis of proteins A, B, and C; and (3) an increase in total phosphorylation amounting to approximately one third to half that induced in K^+-activated or fertilized oocytes but which is qualitatively different. Combined additions of TPA (20–200 n*M*) and different amounts of K^+ (5–30 m*M*) revealed a synergistic effect of these two agents with GVBD occurring within 20 min at K^+ concentrations inefficient without TPA. Also, the external Ca^{2+} concentration required for activation by K^+ ions is reduced when TPA is added. Increased $^{45}Ca^{2+}$ uptake by the oocytes occurs within 5 min following K^+ or sperm-induced activation. No uptake is seen over a 30 min period with TPA. Subthreshold amounts of K^+ ions cause a moderate increase of $^{45}Ca^{2+}$ uptake. This is not further enhanced with TPA concentrations triggering GVBD.

Our results suggest that protein kinase C activation, coupled to increased Ca^{2+} influx, may be involved in the meiosis reinitiation of *Spisula* oocytes.

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Protein kinase C and germinal vesicle breakdown in Spisula. WILLIAM R. ECKBERG (Howard University) AND ETE Z. SZUTZ.

We examined protein phosphorylation in relation to germinal vesicle breakdown (GVBD) in *Spisula* oocytes. Oocytes were labeled with [^{32}P] phosphate and patterns of protein phosphorylation were determined before and at intervals after fertilization until GVBD. GVBD was preceded by an increase in phosphorylation of several proteins. Specific phosphorylation of new polypeptides was observed 3–5 min after fertilization or artificial activation by excess K^+. Oocytes treated with phorbol esters and protein kinase C antagonists were used to study the involvement of protein kinase C in the initiation of GVBD in this species. The tumor-promoting phorbol ester, tetradecanoylphorbol-13-acetate (TPA), triggers GVBD in the absence of excess K^+ and accelerates K^+-induced GVBD at K^+ concentrations from 40 m*M* to 160 m*M*. Three structurally different antagonists of protein kinase C, N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide (W-7), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), and retinol, all block GVBD when induced by fertilization or TPA. The TPA analog, 4α-PDD, does not stimulate protein kinase C, or trigger GVBD. These results suggest that protein kinase C activity is responsible for GVBD when induced by TPA and is involved in GVBD when induced by fertilization. None of the protein kinase C antagonists, except W-7, inhibits GVBD when induced by excess K^+, suggesting that protein kinase C is not required for K^+-induced GVBD. TPA neither accelerates nor delays GVBD when induced by fertilization, further supporting the hypothesis that protein kinase C activity is involved in GVBD when induced by fertilization. Induction of GVBD by TPA requires extracellular calcium. These results strongly indicate that activation of protein kinase C is involved in triggering GVBD in this species.

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Monoclonal antibodies against a 46 kD-protein of the calcium transport system inhibit mitosis in sea urchin embryos. MATHIAS HAFNER AND CHRISTIAN PETZELT (German Cancer Research Center, Heidelberg, Federal Republic of Germany).

To examine calcium function in mitosis, we injected sea urchin (*Lytechinus pictus*) embryos with a monoclonal antibody (7/13) against the mitotic calcium transport system of sea urchin eggs. This antibody specifically labels vesicles of the mitotic apparatus (MA), inhibits calcium uptake of the isolated membrane fraction, and reacts with a 46 kD-protein (Petzelt and Hafner 1986, *Proc. Natl. Acad. Sci.* **83**: 1719–1722).

Two cell embryos were microinjected (Hiramoto 1962). One blastomere was injected, the other served as control. Cells were injected before nuclear envelope breakdown (NEB) and at metaphase. Twelve to twenty picoliters (pl) were injected which represents 2.2–4.8% to the egg volume (500 pl). Antibodies were purified with anti-mouse IgM-agarose and resuspended in a buffer (100 m*M* potassium aspartate; 10 m*M* Hepes, pH 7.0). Antibody purity was tested by SDS-gel-electrophoresis.

Using polarized light microscopy we monitored the effect of injected antibody on the birefringence (BF) of the MA. Within 45 s after injecting the antibody (2–4 mg/ml in the stock) the BF of the MA began to decrease. Loss of BF was first observed at the injection point and subsequently spread over the MA. After 90 s MA BF was completely gone. Injected blastomeres never divided, although two nuclei were occasionally formed. If cells were injected before NEB the nuclear envelope remained intact and no MA was formed.

The non-injected sister blastomere continued normal mitosis and developed to a blastula. Control injections of unrelated monoclonal antibodies (2–10 mg/ml in the stock) or buffer did not effect NEB, MA BF, or progress through mitosis and subsequent cell divisions.

Rapid disruption of spindle fibers but not cytoplasmic microtubules also occurred in tissue culture cells (PtK_2) after microinjection of the monoclonal antibody 7/13, determined by anti-tubulin immunofluorescence.

These results support the idea that calcium ions function in the regulation of mitosis. However, the multifaceted activity of calcium ions currently prevents unequivocal discrimination between primary and secondary consequences. Nevertheless, the rapid and apparently complete disruption of MA microtubules by the antibody 7/13 suggest that calcium may directly cause microtubule disassembly.

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Properties of acrosome activating factor from Spisula *oocytes.* KEITH IVENS (Howard University), HIROSHI UENO, SHELDON J. SEGAL, AND S. S. KOIDE.

We have demonstrated that a component obtained from *Spisula* oocytes induces the acrosome reaction in *Spisula* sperm. Physicochemical characterization of this acrosomal activating factor (AAF) was performed to better understand the mechanism of fertilization.

Spisula oocytes were incubated in a medium containing 1 *M* urea, 10 m*M* EDTA in 5 m*M* Tris-HCl buffer, pH 7.5. AAF activity was assayed by two methods. (1) Fertilizability of *Spisula* oocytes. This method is based upon the principle that reacted sperm are no longer capable of inducing germinal vesicle breakdown (GVBD). Sperm were incubated with the test solutions for 5 min, and added to freshly prepared oocytes. The percent of GVBD was scored 30 min after the insemination. Fractions containing AAF activity inhibit GVBD. (2) Direct determination of the acrosomal reaction. Sperm incubated with and without AAF were fixed in 4% paraformaldehyde solution for 1 h. The percent of sperm that had undergone the acrosome reaction was determined from sperm count using an Axiophoto microscope with video-enhancement (Zeiss). Results from both assay methods were comparable and used interchangeably.

AAF was resistant to heating (60°C for 30 min, 100°C for 10 min), to acid treatment (1 *M* HCl for 20 h at 37°C), and to chemical deglycosylation (trifluoromethanesulfonic acid). It was hydrolyzed by proteinase K but resisted pepsin and trypsin treatment. AAF does not bind to ConA-Sepharose nor to activated charcoal. The factor is soluble in 50% ethanol but precipitated in 95% ethanol. AAF requires Ca^{++} ion to induce the acrosome reaction. AAF preincubated in Ca^{++} containing medium does not induce the acrosome reaction of sperm suspended in Ca^{++}-free ASW, suggesting that Ca^{++} ion does activate AAF directly. *Spisula* AAF does not induce the acrosomal reaction in sperms of sea urchin (*Arbacia*), starfish (*Asterias*), parchment worm (*Chaetopterus*), and horseshoe crab (*Limulus*). In testing horseshoe crab, sperm media with and without K^+ were examined. Estimation of the Mr of AAF was performed by ultrafiltration. AAF activity was associated with the filtrate when 10,000 M.W. cut off membrane (YM10) was used. The activity was retained when 5000 M.W. cut off membrane (YM05) was used, suggesting that the Mr is in the 5000 to 10,000 dalton range. On gel filtration through Sephadex G-50, the major activity was associated with the void fraction, suggesting that aggregation of AAF occurs.

Our present results indicate that AAF is a protein with an estimated Mr in the vicinity of 5000 daltons and probably lacks carbohydrate moieties.

K.I. is an American Society of Cell Biology fellow. This work was supported in part by a grant from the Rockefeller Foundation (GA PS 8615).

Effect of sulfasalazine derivatives on the ability of sperm to fertilize oocytes in Spisula. MUKESH K. SAHNI (Population Council), HIROSHI UENO, S. J. SEGAL, AND S. S. KOIDE.

Men afflicted with ulcerative colitis or regional ileitis and treated with sulfasalazine may develop infertility associated with a deficiency in sperm production. The present study was undertaken to determine the effect of this drug and its derivatives on the fertilizing capacity of *Spisula* sperm.

A quantitative assay based on the percentage of GVBD induced in oocytes exposed to sulfasalazine-treated sperm was used. Dry "sperm" were centrifuged at 1000 × *g* for 5 min. Aliquot of 50 μl of the pellet were suspended in one ml of FSW containing varying concentrations of sulfasalazine derivatives. The treated sperm pellets were diluted 1:20,000 and used to fertilize about 2.5×10^4 oocytes suspended in one ml of FSW. Cultures were scored for GVBD after 80 min.

Sulfasalazine and five derivatives were obtained from Pharmacia AB. The percent fertilization was 97.5, 90.0, 1.7, 93.0, 83.0, 89.7, and 91.0, respectively, with the control, sulfasalazine, Ph CL 68A, Ph CH 74A, Ph CK 61A, Ph CL 06A, and Ph CL 42A, at a concentration of 500 μM. The dose-response of Ph CL 68A, a chlorinated aromatic ketone, was determined. The percent fertilization was 97.0, 83.0, 79.0, 4.7, 1.0, and 0.8, respectively, with control, 100, 200, 300, 400, and 500 μM of the drug. When the assay system contained 5% bovine serum albumin (BSA) and 300 μM of Ph CL 68A, 95.2% of the oocytes had undergone

GVBD compared with 2.9% when BSA was omitted, showing that BSA nullified the inhibitory effect of the drug.

To determine the metabolic pathway influenced by Ph CL 68A, its effect on O_2 uptake of *Spisula* sperm and protein kinase activity of the catalytic subunit obtained from bovine heart (Sigma Chemical Co.) was determined. The O_2 uptake by *Spisula* sperm was inhibited by 20% and 78% at concentrations of 215 and 360 μM of the drug, respectively, suggesting that it acts by depressing mitochondrial respiration and oxidative phosphorylation. Protein kinase activity was completely inhibited by Ph CL 68A at 100 μM, while gossypol at 100 μM sustained only a 17% inhibition of the enzyme.

In conclusion, Ph CL 68A, a sulfasalazine derivative, inhibited *in vitro* fertilization of *Spisula* oocytes. Its probable mode of action is to suppress O_2 uptake by *Spisula* sperm and inactivate protein kinase.

M.K.S. is a Rockefeller Foundation fellow. The sulfasalazine derivatives were provided by Dr. J. Carlsson, Pharmacia AB, Sweden.

Surface and internal movements in fertilizing Phallusia *eggs.* C. SARDET, S. INOUÉ, L. F. JAFFE, AND J. E. SPEKSNIJDER (Marine Biological Laboratory).

Phallusia mammillata is a European ascidian with exceptionally clear eggs in which öoplasmic segregation and mitosis can be well studied *in vivo.* We denuded eggs using 0.1% trypsin for 2 hours and synchronously fertilized them with sperm preactivated with chorionated eggs. Several successful video sequences were obtained using slightly flattened eggs observed with rectified DIC and polarization optics recorded on an optical memory disc or a conventional time lapse recorder.

In one remarkable sequence, we saw a contractile wave start immediately after fertilization at the animal pole and move to the vegetal one. Sawada has reported similar waves in dechorionated *Ciona* eggs (1981, *Roux's Archives* **190**: 208–214). In both species this wave travels at about 2½ μm/s over most of the egg. The first and second polar bodies then form at about 5 and 20 minutes after fertilization. In between we saw a dozen pulsating movements consisting of alternative protrusions and retractions at the animal pole with a period of about 1.0 minutes. Soon after second polar body formation, a hitherto undescribed protrusion—quite reminiscent of spiralian polar lobes—forms at the vegetal pole and persists for several minutes.

The myoplasm—which first becomes visible as a refractile zone under the vegetal pole—begins its posterior movement soon after second polar body formation. This process occurs in two discrete phases: during the first (which is accompanied by posterior movement of the sperm aster), a dozen pulsatile contractions occur at the vegetal pole, again with a period of about 1.0 minute; during the second phase, the myoplasm rapidly moves into the well-known posterior crescent as the sperm aster moves inward to meet the female aster.

Moreover, we now have evidence that the sperm may enter the animal hemisphere before cortical contraction carries it to the vegetal pole.

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Calcium pulses and waves through ascidian eggs. J. E. SPEKSNIJDER, D. W. CORSON, L. F. JAFFE, AND C. SARDET (Marine Biological Laboratory).

It has been postulated that activation of all eggs on the vertebrate line involves a large, internally supported wave of free calcium ions (Jaffe 1983, *Dev. Biol.* **99**: 265–276). Using aequorin-loaded *Ciona* eggs we have measured a large free calcium pulse during activation (Speksnijder *et al.* 1986, *Biol. Bull.* **170**: 542). Using an imaging photon detector we now have seen that this pulse travels across these eggs at about 7–9 μm/s at 15°C. Moreover, the remarkably clear (and chemically dechorionated) eggs of *Phallusia mammillata* likewise show a large traveling pulse of free calcium moving over much of the egg at approximately 5–7 μm/s at 21°C. This pulse rises about 1000-fold above instrumental background suggesting a peak free calcium level of about 3 μM instead of the 10 μM which we now estimate for *Ciona* (where the rise is about 10,000-fold).

As soon as the luminescence level returns to background—3–4 minutes after the activation pulse starts—a series of 12 to 16 postactivation pulses begins. This series starts at first polar body formation, 4–5 minutes after fertilization, and ends at second polar body formation at 22 minutes. The peak levels of these postactivation pulses are about 10-fold lower than the activation pulse and thus may represent a rise to about 1 μM. The later pulses in this series are often double with a second, smaller component peaking about 20 seconds after the first. These postactivation pulses start locally and then often travel across the egg. Successive pulses either start in the same region or in an antipodal one. The postactivation calcium pulses are sufficiently similar in stage, number, and period to the contractile pulses seen at the animal pole—see companion abstract—as to suggest a close relationship.

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Effect of gossypol on protein phosphorylation in Spisula *sperm.* Hiroshi Ueno (Rockefeller University), Sheldon J. Segal, and S. S. Koide.

Gossypol, a yellow polyphenolic compound isolated from cottonseed, inhibits sperm motility. This inhibitory action of gossypol has not been clarified. Gossypol inactivates enzymes present in sperm mitochondria, and reduces the production of ATP. Since sperm motility is totally dependent upon the supply and utilization of ATP, gossypol may act by influencing ATP metabolism. Sperm motility also is dependent upon phosphorylation-dephosphorylation of specific protein components, *i.e.,* protein phosphorylation triggers motility while motility ceases on protein dephosphorylation. Thus, the present study examined the effect of gossypol on protein phosphorylation of *Spisula* sperm.

Freshly prepared *Spisula* sperm was suspended in MBL artificial seawater (ASW) and its oxygen consumption was measured by using YIC oxygen meter (Clark type O_2 electrode, Yellowstone Instrument Co.). Linear consumption of oxygen was recorded over 30 min. In the presence of 180 μM gossypol the oxygen consumption was reduced by 70% of the control value. At lower concentration (45 μM) there was only a slight reduction of the consumption (less than 5%). The present results suggest that gossypol clearly affects oxygen consumption of sperm probably by blocking oxidative phosphorylation in the mitochondria.

To determine protein phosphorylation of sperm, sperm suspension (2×10^8 cells/ml) was incubated with (γ-P^{32}) ATP (250 μCi, 0.085 nmol) in the presence and absence of gossypol (100 μM) for 60 min at 25°C. The phosphorylated proteins were extracted by suspending the sperm pellet in a medium containing 0.15 M KCl, 1 mM dithiothreitol, 0.1 mM $CaCl_2$, 0.04% Triton X-100 (v/v), 2 mM Tris buffer, pH 8.2, for 5 min at room temperature. The homogenate was dialized against water and analyzed by SDS-PAGE. The proteins in the gel were stained with Coomassie blue. No significant change in the protein pattern was found. On autoradiography (Kodak X-Omat AR film, 3 days with Du Pont Enlightning screen), a significant decrease in the intensity of the radiolabeling of the protein bands corresponding to estimated Mr of 160 K, 142 K, 97 K, 80 K, 55 K, and 43.5 K was observed.

Our results suggest that gossypol inhibits oxidative phosphorylation of *Spisula* sperm and blocks phosphorylation of several sperm proteins. The data supports the thesis that gossypol inhibits sperm motility by blocking ATP production and utilization and that sperm motility may be controlled by phosphorylation-dephosphorylation of specific proteins.

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NEUROBIOLOGY

External calcium and the closing of sodium channels in squid giant axons. Clay M. Armstrong and Wattana Bamrungphol (Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania).

As reported previously, K channels in squid neurons lose their ability to close and their selectivity when external calcium concentration is below 5–6 mM. Here we report the effects of low calcium concentrations on Na channels in squid giant axons. To eliminate interference from K channels, we (1) functionally destroyed all K channels by perfusing internally and externally for 30 minutes with K-free solutions, and (2) included 7 mM Ba ion in the internal test solution (225 K glutamate, 50 KF, 7 $BaCl_2$, sucrose, Hepes). Neither altered the Na channel behavior significantly. Lowering calcium from 20 to 5 mM (525 NaCl, 5 $CaCl_2$, Hepes) leads to an initial increase in sodium current amplitude, followed by a progressive decrease and eventual disappearance. The time constant of Na channel closing, determined by returning membrane potential to −70 mV after a short pulse, was affected little by calcium changes from 50 to 10 mM, and increased steeply following an approximately hyperbolic curve for calcium below 5–8 mM. The results are consistent with the hypothesis that calcium ions are essential for closing of Na channels, and that when calcium falls below the required level, many channels remain open for a time and then inactivate or 'slow inactivate,' with the result that current magnitude decreases. There is no evidence that Na channels lose their selectivity in low calcium.

Potassium channel blockers reduce transmitter release at the squid giant synapse. George J. Augustine, Milton P. Charlton, and Richard Horn (Section of Neurobiology, Department of Biological Sciences, University of Southern California).

The model of Stanley and Ehrenstein (1985, *Life Sciences* **37**: 1985–1995) proposes that Ca-dependent neurotransmitter release is due to activation of Ca-dependent K channels present in synaptic vesicle membranes. To test this hypothesis we asked whether substances which block Ca-dependent K channels influence transmitter release at the squid giant synapse. The quaternary ammonium compounds tetrapentylammonium bromide (or chloride) (TPA), N-nonyltriethylammonium bromide (Cg), and decamethonium were microinjected into giant presynaptic terminals of *Loligo pealei,* while postsynaptic electrical responses were used as an assay for transmitter release. Pressure or iontophoretic injection of minute quantities of any of these three compounds caused a rapid, reversible reduction in postsynaptic responses elicited by presynaptic action potentials. Injection of tetraethylammonium chloride (TEA), a quaternary ammonium derivative which is relatively ineffective in blocking Ca-dependent K channels from the cytoplasmic surface, did not block release. Reductions in release were not due to reduced amplitude or duration of presynaptic action potentials; on the contrary, these agents often increased the duration of presynaptic action potentials, presumably by blocking delayed rectifier K channels responsible for action potential repolarization. We tested whether these compounds block release via an action on presynaptic Ca channels by using the 3-microelectrode voltage clamp method to measure Ca currents during injection of these compounds. Na and K channel currents were blocked with drugs thus providing uncontaminated measurement of Ca channel currents. Both TPA and Cg blocked Ca currents and transmitter release concurrently, suggesting that their effects on release may be a consequence of decreased entry of Ca into the presynaptic terminal. We conclude that injection of these compounds does not provide a definitive test for the hypothesis. On the other hand, these compounds may prove interesting as organic blockers of presynaptic calcium channels.

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Development of circadian rhythms in the Limulus *visual system.* ROBERT B. BARLOW, JR. (Syracuse University).

A circadian clock is an integral part of the visual system of the horseshoe crab, *Limulus polyphemus.* Located in the brain, the clock transmits efferent optic nerve activity to all major visual organs at night. Its input to the lateral compound eyes generates pronounced circadian rhythms in the anatomy, physiology, and metabolism of the retina. The multiple rhythms combine to increase retinal sensitivity as much as 100,000 times at night (Barlow *et al.* 1980, *Science* **210:** 1037).

How do the circadian rhythms develop? Are they genetically inherited or environmentally programmed? To answer these questions, we removed eggs from nests within minutes of fertilization and raised them in the laboratory under three conditions: cyclic lighting (LD), constant light (LL), and constant dark (DD). Most eggs hatched after about one month. After about 6 months the animals reached the third juvenile stage (4 to 5 mm across the carapace). At this third stage stable electroretinographic responses (ERGs) could be recorded from the lateral eyes for about one month. The eyes measured about 200 μm and contained about 50 ommatidia. Endogenous changes in the amplitude of the ERG were used as an indication of a circadian rhythm in retinal sensitivity (Barlow 1983, *J. Neurosci.* **3:** 856).

Most all LD animals exhibited circadian rhythms in ERG amplitude, but LL animals did not. DD animals had degenerate eyes which did not yield recordable ERGs. Circadian rhythms could be generated in most LL animals by exposure to multiple light/dark cycles and in some LL animals by exposure to a single 12 h light pulse.

I conclude that the development of a circadian rhythm in lateral-eye sensitivity requires cyclic light from the environment, but the length of its period does not. Period length appears to be determined genetically. Environmental lighting may trigger the circadian rhythm by synchronizing the activity of multiple oscillators.

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Comparative organization of supramedullary neurons in toadfish, spiny boxfish, and puffer. M. A. BARRY (Marine Biological Laboratory), M. WEISER, R. BAKER, AND M. V. L. BENNETT.

Supramedullary neurons (SMNs) in toadfish (*Opsanus tau*), spiny boxfish (*Chylomycterus* sp.), and a freshwater puffer (*Tetrodon* sp.) were studied with intracellular recording and injection of horseradish peroxidase. Toadfish SMNs are small, numerous, and have a single small axon with few branches. Axons of the most rostral SMNs course rostrally to exit the brain in the vagal or trigeminal roots. Axons of more posterior SMNs exit the spinal cord in a dorsal root at the level of the SMN cell body. Boxfish and puffer SMNs are much larger and fewer in number. Boxfish SMNs have large single axons that branch intraspinally and have neuritic arborizations that may represent sites of synaptic input. Often branches of single SMN axons exit the brain in both the trigeminal nerve and dorsal roots. Puffer SMNs have numerous axons that exit via multiple dorsal roots and the trigeminal nerve. These processes can be followed into the skin where they apparently terminate. In all species, impulses can be synaptically evoked by tactile stimulation anywhere on the skin. In toadfish and boxfish, visual stimulation and saccular movement are also effective. As previously shown in a puffer (Bennett *et al.* 1959, *J. Gen. Physiol.* **43:** 159), toadfish SMN axons are efferent and their volleys can be recorded from dorsal roots. Discharges of toadfish SMNs are less well synchronized than in puffer, but the degree of synchronization, as well as intracellular recording of small fast depolarizations during synaptically and antidromically evoked activity, suggests that they too are electrotonically coupled. Because of coupling, small cells with single axons as in toadfish may be equivalent to large cells with multiple or branched axons as in puffer and boxfish. Despite differences in morphology and degree of synchronization it is likely that these SMNs are homologous and have a similar but still unknown function.

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Two types of adenosine receptors on frog motor nerve terminals mediate opposite effects on transmitter release. SUSAN R. BARRY (University of Michigan).

Adenosine triphosphate is released with acetylcholine from motor nerve terminals and may be hydrolyzed to adenosine in the synaptic cleft. Adenosine (2 μm to 1 mM) usually depresses transmitter release from the frog neuromuscular junction. However, at high concentrations (50 μM to 1 mM), adenosine enhances transmitter output in about 5% of preparations. These two effects of adenosine may be mediated by two different adenosine receptors on motor nerve terminals which resemble the A1 and A2 receptors found in other tissues.

L-N^6-Phenylisopropyladenosine (L-PIA) is more potent than adenosine at A1 receptors but less potent at A2 receptors. 5′-N-Ethylcarboxamide-adenosine (NECA) is equipotent with adenosine at A1 receptors but more effective at A2 receptors. At A1 receptors, adenosine depresses while, at A2 receptors, adenosine enhances the activity of adenylate cyclase.

Effects of L-PIA and NECA were tested at the frog cutaneous pectoris neuromuscular junction. Changes in transmitter release were measured as changes in the frequency of spontaneous miniature endplate potentials (mepps). L-PIA was 1000-fold more potent than adenosine in depressing mepp frequency. In contrast, NECA (1 μM) produced a transient increase followed by a sustained decrease in mepp frequency. The initial enhancement of mepp frequency was prolonged in the presence of the phosphodiesterase inhibitor, papaverine (0.5 mM).

The decrease in transmitter release produced by adenosine may be mediated by A1 receptors since L-PIA was more potent than adenosine in depressing mepp frequency. Adenosine may enhance transmitter output by binding to A2 receptors since NECA caused a transient increase in mepp frequency. In other tissues, adenosine stimulates adenylate cyclase at A2 receptors. The transient increase in mepp frequency produced by NECA may result from a transient increase in cyclic AMP. Thus, NECA produced a sustained enhancement of mepp frequency in the presence of papaverine, a phosphodiesterase inhibitor, perhaps because papaverine prevented the breakdown of cyclic AMP.

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Cells postsynaptic to barnacle photoreceptors are sensitive to GABA. JOSEPH C. CALLAWAY (University of Washington, Seattle) AND ANN E. STUART.

Several lines of evidence suggest that GABA is the transmitter released by barnacle photoreceptors (PRs) onto postsynaptic cells (I-cells). The PRs can synthesize 3H-GABA from injected 3H-glutamate and transport it to the cell's presynaptic terminals; furthermore, 3H-DABA is taken up selectively by receptor terminals (Koike and Tsuda 1980, *J. Physiol.* **305:** 125–138). Koike (1983, *The Physiology of Excitable Cells,* Alan R. Liss. Pp. 523–534) has also reported a small light-induced release of injected 3H-GABA from the PRs. On the other hand, Timpe and Stuart (1984, *Brain Res.* **307:** 225–231) could not block synaptic transmission with picrotoxin or bicuculline, measure significant amounts of endogenous GABA in PR axons, or see a consistent effect of 10-4M GABA on the I-cell when applied in the perfusate.

We find that 10-3M GABA in the bath hyperpolarizes I-cells of *Balanus nubilus* 5 to 10 mV and reduces their responses to light. When applied by pressure onto an I-cell's soma, GABA (1 M in the microelectrode) hyperpolarizes the cell by increasing its conductance. The reversal potential of this hyperpolarization is negative to the cell's −40 to −50 mV resting potential and dependent on external K, as is the reversal of the cell's response to light. The GABA response persists in a saline of low (2.5 mM) Ca and (12.5 mM) Co that blocks synaptic transmission from the PRs; thus the GABA receptors are on the I-cell itself. In this low Ca saline with Co the response triples or quadruples in amplitude, but it is abolished in Ca-free saline, indicating a sensitivity of the GABA receptor to divalent cations. The response is insensitive to picrotoxin and bicuculline. Thus, in its ion selectivity and pharmacology, the action of pressure-applied GABA on the I-cell is consistent with that of the PRs' transmitter.

The reversal potential for pressure-applied GABA is less negative than that for the light response (the PRs' transmitter), suggesting that the receptors mediating the GABA response are on the cell soma rather than at electrically distant postsynaptic sites in the neuropil.

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Horizontal cell responses to modulated stimuli in the skate retina. RICHARD L. CHAPPELL (Hunter College, CUNY, New York), KEN-ICHI NAKA, HIROKO M. SAKAI, AND HARRIS RIPPS.

The skate (*Raja erinacea* and *R. oscellata*) is the only vertebrate known to have but a single class of photoreceptors, namely rods. Nevertheless, its horizontal cells, as well as other retinal neurons, have the remarkable ability to recover sensitivity (*i.e.*, adapt) after exposure to intensities which initially block all light-evoked responses. The system is uniquely suited to the study of dynamic properties of horizontal cell responses without the complication of the cone input of duplex retinas.

To study response dynamics under conditions of ambient illumination more nearly like those encountered in the normal environment, we used as photic stimuli sinusoidal and Gaussian white noise modulation of the mean intensity. We find that over a range of five log units in mean intensity, a dynamic steady state is established. Thus, although the horizontal cell adapts slowly to the mean intensity of the modulated stimulus train, the responses to sinusoidal or white-noise transients become stable, and responses to equivalent incremental and decremental "flashes" are nearly mirror images of each other.

However, a rather surprising result was obtained in analyzing the steady-state responses at various luminance levels. For the brightest intensities (covering a range of three log units up to a mean of 20 $\mu W/cm^2$), response amplitude depended solely on contrast, and was independent of the mean luminance. That is, contrast sensitivity remained unchanged as the mean luminance underwent a 1000-fold increase. Incremental sensitivity, on the other hand, was inversely porportional to the mean (*i.e.*, obeyed the Weber-Fechner law) over this luminance range. Clearly, then, the main adaptational feature of the skate retina is its ability to maintain a stable contrast sensitivity despite exposure to large changes in ambient illumination.

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Modulation of ionic channels by somatostatin in pituitary cells. GABRIEL COTA AND CLAY M. ARMSTRONG (Department of Physiology G4, University of Pennsylvania).

We studied the effect of somatostatin (S-14) on Ca and K currents in clonal pituitary GH3 cells, using whole-cell recording with patch pipettes at 20–23°C. Bath application of S-14 (200 ng/ml) from a delivery pipette placed close to the cell induced a drastic reduction of inward Ca currents. Two different populations of Ca channels, SD and FD, are present in GH3 cells (Armstrong and Matteson 1985, *Science* **227**: 65–67). S-14 preferentially inhibits by 50–90% the current carried by low-voltage-activated, SD channels. This action of S-14 is rapid and persistent: half-maximal inhibition of Ca currents is attained within 1 min and little recovery is observed after 20 min of continuous washing. S-14 also affects K channels. Outward K currents recorded from cells internally dialyzed with 10 or 20 mM EGTA have two main components. A fast, transient component (TK current) can be removed by applying a 100 ms-prepulse to 0 mV. The remaining current is a slower, long-lasting component (LK current). In the present of S-14 the LK current is reduced by about 50%, whereas the TK current is little affected. Inhibition of the LK current by S-14 does not seem to be causally linked to inhibition of SD Ca channels since recovery of the K current is almost complete within 20 min of washing. At present the functional role of S-14 action on the LK current is not clear. However, inhibition of Ca currents may contribute to inhibition of hormone secretion by somatostatin.

Calcium currents in paramecium are blocked by one class of calmodulin antagonists. BARBARA E. EHRLICH (University of Connecticut Medical Center), AVRUM COHEN, AND MICHAEL FORTE.

Paramecium are the most primitive eukaryotes known to have voltage-dependant calcium channels. These channels are similar to at least one class of metazoan calcium channel in that they are insensitive to the organic calcium channel blockers verapamil, diltiazem, and the dihydropyridines. The only compounds known to block the paramecium channels are the naphthalene sulfonamide calmodulin antagonists (*e.g.*, W-7). Other calmodulin antagonists are ineffective. We tested an analogue of W-7, which we have named W(12)Br, on two species of paramecium and on calcium channels that have been incorporated into planar lipid bilayers. The two species of paramecium studied were *P. tetraurelia*, the commonly used fresh water species and *P. calkinsi*, a marine species found in the tide pools of Woods Hole, Massachusetts. A behavioral assay was used in the initial tests of W(12)Br which measured the duration of backward swimming after a potassium-induced depolarization. This simple assay correlates well with direct measurements of calcium currents in intact voltage-clamped paramecium. In both species 1 μM W(12)Br leads to complete block and ~ 0.1 μM reduces the response to half. We also measured the potency of W(12)Br on single channel currents. In these experiments ciliary membrane vesicles harvested from *P. tetraurelia* were incorporated into bilayers made at the tip of patch-style pipettes. Complete inhibition of the bilayer currents was obtained with 1 μM W(12)Br. When 0.1 μM W(12)Br was tested, channel open times decreased to 30% of control and very long silent periods began to appear. The single channel conductance was unchanged. In both series of experiments, W(12)Br was 100 times more effective than W-7. Since these compounds are effective in blocking channels in the cell-free system, it seems unlikely that they are working via calmodulin. However, the mechanism of action is likely to be complex since more than one channel parameter is altered by W(12)Br.

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Aversive conditioning of eye extension in the green crab, Carcinus maenas. RICHARD D. FEINMAN AND CHARLES I. ABRAMSON (State University of New York Health Sciences Center at Brooklyn, Brooklyn, New York 11203).

Extension of the green crab eye, following reflex withdrawal, was suppressed by a punishment procedure in which every elevation of the eye of experimental animals was punished with a 0.5 s puff of air to the eye. Yoked controls received a puff of air at the same time as the experimental (master) animals independent of their behavior or eye position. In a second control, animals were punished for every extension, but after a 10 s delay. For the yoked experiments, eight single animals in which one eye was the master eye and the other, the yoked control, were more reliable subjects than eight pairs of master and yoked animals with one eye of each being punished. Three sessions of 30 min, separated by 12 h, were used. Learning in the yoked paradigm was indicated by: (1) reduction in the response rate of all experimental animals. (2) Increase in the latency of masters compared to controls to extend the eye after punishment. (3) Extension of the yoked eyes for long periods during the training session. (4) Increase in the probability of the yoke eye being raised before the master after punishment. The delayed punishment control further supported the hypothesis of learning by experimental animals. Eight animals subjected to punishment after a 10 s delay showed numerous responses, and no indication of learning despite the fact that they received many more punishments than experimentals. In summary, contingent punishment (as compared to non-contingent, or delayed punishment) can repress elevation of the eye of the green crab, *Carcinus maenas*, providing an invertebrate model for a simple form of operant conditioning. For the yoked paradigm, a single animal with one eye serving as a control for the other is a more reliable and reproducible preparation than two separate animals.

Primary and secondary vestibular neuron organization in the winter flounder, Pseudopleuronectes americanus. W. GRAF AND R. BAKER (Marine Biological Laboratory).

Previously, we demonstrated that the adaptive changes in the vestibulo-ocular reflex (VOR) system of the adult (post-metamorphic) flatfish could be explained by the contralaterally projecting second-order vestibular neurons of the left (downside) horizontal semicircular canal (Graf and Baker 1985, *J. Neurophysiol.* **54:** 900–916). The present study investigated the bilateral symmetry of the system, especially the existence of ipsilaterally ascending axons, as well as the projections of primary vestibular afferents.

Single cell morphology of second-order vestibular neurons linked to one or the other horizontal canal, visualized with the intracellular horseradish peroxidase (HRP) injection method, revealed a qualitatively bilaterally symmetric distribution of neurons with terminals in either both superior rectus and inferior oblique motoneuron pools or with the antagonists to these muscles, the inferior recti and superior obliques (trochlear nuclei). Surprisingly, all second-order neurons had contralaterally ascending main axons, and some of the cell somata of stained neurons were found contralateral to the stimulated horizontal canal. The latter organization, in particular, has never been observed in mammalian VOR systems. Extra- and intracellular HRP data on primary vestibular afferents offered no cues for this arrangement. As described in other teleost fishes, primary afferents from the semicircular canals terminated in the five subnuclei of the vestibular complex (anterior, magnocelluar, descending, tangential, posterior), and additionally in the eminentia granularis and the medial reticular formation. In particular, axon collaterals did not cross the midline to provide possible monosynaptic input to the contralaterally located vestibular nucleus neurons. In summary, our data indicate a bilaterally symmetric distribution of post metamorphic VOR neurons of both excitatory and inhibitory character with absence of ipsilaterally ascending neurons. However, in light of an inconspicuous primary vestibular afferent system, possible axo-axonal and electrotonic contacts between primary afferents and second-order neurons, or among second-order neurons need to be investigated to explain the activation of contralateral second-order neuron somata by monosynaptic potentials.

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Identification of Drosophila *genomic clones with homology to the voltage-sensitive sodium channel from* Electrophorus. L. M. HALL (Albert Einstein College of Medicine, Bronx, New York), D. W. GIL, J. KEEN, F. R. JACKSON, S. SUPATTAPONE, T. J. ZUMBROICH, AND K. R. BLEY.

A number of temperature-sensitive paralytic mutants in *Drosophila melanogaster* affect saxitoxin binding and/or propagation of action potentials. To determine which of these mutants define genes for structural components of the voltage-sensitive sodium channel, we cloned the *Drosophila* gene(s) homologous to evolutionarily conserved sequences of the cDNA for this channel from *Electrophorus*. We prepared a 38 base oligonucleotide probe from the S5 membrane-spanning region closest to the carboxy terminal end of the *Electrophorus* channel (Noda *et al.* 1984, *Nature* **312**: 121–127). The region shows only three base changes when the cDNA sequences from *Electrophorus* and rat are compared, provided that deoxyinosine is used in the wobble position for degenerate codons. Using hybridization conditions that require at least 20 matching base pairs, we identified 5 genomic clones that fall into 2 classes based on restriction mapping and *in situ* hybridization to polytene salivary gland chromosomes. The D class has 1 representative clone and hybridizes to 61F1-4 on the left arm of chromosome 3. No putative sodium channel mutants have yet been identified in this region. The C class has 4 representative clones and maps to 64A1-5 on the left arm of chromosome 3. The temperature-sensitive paralytic mutant *tip-E* has been cytogenetically localized to 63A-64C, a region which includes the C clone area of hybridization. The saxitoxin-binding component in *tip-E* is abnormally labile *in vitro* (Jackson *et al.* 1986, *J. Neurogenet.* **3**: 1–17). This increased lability coupled with the tentative colocalization of *tip-E* and the C clones lead us to postulate that *tip-E* represents a gene coding for a sodium channel structural component homologous to those of *Electrophorus* and rat.

The incorporation of serine into phospholipids of the brain and giant fiber system of the squid. P. G. HOLBROOK (Department of Applied Biological Sciences, E25-617, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139) AND R. M. GOULD.

We investigated incorporation of ^{3}H-serine into phospholipids of squid brain, stellate ganglion, and axoplasm extruded from the associated giant axon. Radioactivity in lipid extracts was recovered in both phosphatidylserine (PS) and phosphatidylethanolamine (PE) in all three tissues. The serine incorporated into PS by pure axoplasm was inhibited by EGTA (10 mM) and stimulated by calcium (2.5 mM) consistent with its occurrence by a calcium dependent base-exchange reaction mechanism. In axoplasm, the accumulation of label into PE showed a lag, relative to that into PS, consistent with its formation by decarboxylation of PS. When expressed per unit of protein, serine base-exchange activity was much higher in brain and ganglion (41.1 ± 4.8 and 22.6 ± 2.0 picomoles/mg protein/h respectively) than in axoplasm (2.7 ± 0.3 picomoles/mg protein/h). This is probably due to lower amounts of phospholipid in axoplasm (0.6 μg lipid phosphorous/mg protein) than in brain and ganglion (4.1 and 3.0 μg lipid phosphorous/mg protein, respectively). Expressed as units of lipid phosphorous the activities are 10.0 ± 0.1, 7.6 ± 0.7, and 4.9 ± 0.6 picomoles/μg lipid phosphorous/h in brain, ganglion, and axoplasm, respectively. Since much of the membrane associated with axoplasm is in transit to nerve endings, these data provide preliminary evidence that the serine base-exchange enzyme may be transported down axons and incorporated into nerve endings. Alternatively, the activity observed in axoplasm may be associated with the stationary smooth endoplasmic reticulum of the giant axon.

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The circadian clock in the Limulus *brain modifies the electrical properties of the lateral eye's photoreceptors.* E. KAPLAN, (The Rockefeller University, N.Y.) R. B. BARLOW, JR., AND G. RENNINGER.

The lateral eyes of the horseshoe "crab," *Limulus polyphemus,* undergo remarkable circadian rhythms in their structure and function. These changes are caused by the nightly activity of efferent fibers, originating in the brain. In the past we reported that at night the light response from the photoreceptors increases, the frequency of spontaneous discrete waves (quantal bumps) decreases, and that more photons are caught.

We have now recorded from single retinular cells *in vivo* for several days, and found that at night, the time course of spontaneous and light-evoked quantal bumps is altered: the rise time is unaffected, but the decay to resting potential is substantially prolonged. The change in quantal bumps time course takes place without detectable changes in resting potential, membrane resistance, or amplitude distribution of the bumps. The net result from the prolongation of the quantal response is that more current flows across the membrane for each absorbed photon at night: the area under the average night-time bump is 2.4 times the area under the average day-time bump.

These results suggest that the circadian clock can exert a differential effect on some ionic channels in the photoreceptor membrane, without affecting other channels. One possibility is that the clock, which is known to release octopamine in the eye, might partially block voltage-sensitive potassium channels, slowing down the repolarization of the membrane. The change in the photoreceptor's electrical properties and the resulting modification of the response to single photons would alter the frequency response of the eye, trading temporal resolution for maximal sensitivity.

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Voltage dependence of junctional conductance in ascidian embryos. J. A. KNIER, M. W. MERRITT, R. L. WHITE, AND M. V. L. BENNETT (Albert Einstein College of Medicine).

Ascidian embryo blastomeres are electrotonically coupled, and the junctional conductance (gj) is voltage dependent. This property may be involved in establishing communication compartments (see abstract by Merritt *et al.*). As reported previously, transjunctional voltage (Vj) of either sign decreases gj. The steady stage Vj-gj relation is well fit by a Boltzmann equation consistent with open and closed states of series gates, one in either of the apposed membranes. For small Vj steps of a single sign, a single gate is modulated and gj relaxes to its steady state exponentially. At appropriate Vj's, fluctuations should result from stochastic opening and closing of channels. We recorded these fluctuations in two ways. In weakly coupled pairs stepping one cell in voltage clamp changes voltage in the second, unclamped cell only a few percent. At Vj's where gj is at an intermediate value the potential in the unclamped, postjunctional cell shows obvious fluctuations. At lower Vj's where gj is near its maximum value, the fluctuations are smaller even if the postjunctional voltage is larger. The fluctuations also are smaller at larger Vj's where gj is near its minimum and the postjunctional voltage is smaller. Similar maxima in noise are observed in dual voltage clamp where one cell is stepped and the other is held at the resting potential and junctional current is measured directly. Current fluctuations are large at intermediate Vj's where channels should be opening and closing and small at lower and higher Vj's where channels are predominantly open and closed, respectively. In limited analysis of these data the power spectra are fit by Lorentzian curves. Corner frequencies are in the range predicted by time constants of exponential relaxation, but correlation of noise and relaxation in the same cell pairs is required. Variance data are consistent with a single channel conductance in the range of 100pS, similar to values proposed from other experimental systems.

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"Sticky-patch" measurements of currents in nerve terminals of the lizard. CLARK A. LINDGREN AND JOHN W. MOORE (Department of Physiology, Duke University Medical Center, Durham, North Carolina).

Ionic currents were measured in the neuromuscular junction of the dewlap extensor muscle in the lizard *Anolis carolinensis.* The heat-polished tip ($\sim 5\ \mu$ i.d.) of the extracellular glass microelectrode was positioned using high power (640×) Nomarski optics. When pressed gently against terminal branches, the tip became mechanically "stuck" to the underlying membrane and/or connective-tissue sheath, allowing for prolonged and stable recording; however, the seal was not the gigaohm seal normally employed in patch clamps. Currents flowing through Ach receptor channels in the postsynaptic membrane were blocked by bath application of 5 μM pancuronium bromide. In standard saline (130 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$ and 20 mM $NaHCO_3$ saturated with 5% CO_2) a two component positive signal was recorded when an action potential invaded the terminal. The first component was the capacitative current flowing during the rising phase of the action potential. The second was identified as an outward potassium current since it could be reduced by increasing the potassium ion concentration in the bath ($[K]_0$) and blocked by adding 8 m*M* tetraethylammonium (TEA). In TEA, a small inward current was observed. This was identified as a calcium current because it increased in amplitude with increasing calcium concentrations and was abolished when cobalt was substituted for calcium. Addition of 0.1 m*M* 3,4-diaminopyridine (a potassium channel blocker) to 5 m*M* calcium saline caused the original potassium current to be replaced by a slow outward current. This current was delayed at least 1 ms from the capacity current peak and lasted approximately 5 ms. This appears to be a calcium-activated potassium current because it could be reduced by increasing $[K]_0$ and blocked by (a) replacing the calcium with cobalt, or adding (b) 8 m*M* TEA or (c) 50 n*M* Choribdotoxin (CTX).

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The spread of epileptiform discharges in the somatosensory cortex of the marine rat measured with voltage sensitive dyes. J. A. LONDON, M. CATTARELLI, AND L. B. COHEN (Yale University School of Medicine).

The optical recording method was used to study the progression and spatial distribution of epileptiform discharges in the somatosensory cortex of the rat. Wistar SPF, male adult rats were anesthetized with equithesine, paralyzed, and artificially ventilated. The somatonsensory cortex was exposed and stained with the fluorescent styryl dye, RH795, (supplied by R. Hildesheim and A. Grinvald) at a concentration of 1–2 mg/ml applied for 1 h, followed by a 30 min saline wash. The stimulus consisted of brushing a thin nylon filament across mystacial whiskers. Optical signals were recorded via a 12 × 12 element photodiode array. A ball electrode was placed on the somatosensory cortex adjacent to the region of optical recording. In order to measure the optical signal in response to whisker movement in the control preparations, 32 trials were averaged. Epileptiform discharges were then induced by application of 1 m*M* bicucculine to the cortex. After addition of biccuculine signals were recorded in single trials. Two general classes of responses were recorded, those evoked by whisker stimulation and spontaneous events. The evoked response consistently originated from the same site as the control signal, but propagated over a much larger area. The evoked response was stereotypic within the same animal but differed somewhat from animal to animal (25 responses, 12 animals). The spontaneous response was much more variable in origin, propagation direction, and spatial location than the evoked response (16 responses, 7 animals). In addition the electrical activity recorded with the ball electrode was often not well correlated with the optical signals. Optical recording appears to provide additional information about the location and propagation of epileptiform discharges.

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Optical monitoring of potassium accumulation in slices of skate (Raja erinacea) *cerebellum in vitro.* A. L. OBAID, A. KONNERTH, AND B. M. SALZBERG (University of Pennsylvania).

Skate cerebellar slice preparations were used to study the electrophysiology of a relatively simple, well organized brain structure with distinct morphological layers. The molecular layer contains mainly the axons of the granule cells (parallel fibers), dendrites of Purkinje cells, and glial cells. In a coronal slice, the parallel fibers lie in the plane of the slice and are stimulated readily with a bipolar electrode. We used a system for multiple site optical recording of transmembrane voltage (MSORTV) to record electrical activity simultaneously from 124 sites in the molecular layer of 700–1000 μm coronal slices, cut with a Vibroslicer (Campden Instruments, Ltd. Model 752). The pyrazo-oxonol dye RH-155 (Grinvald *et al.* 1980, *Biol. Bull.* **159:** 484), available commercially as NK 3041, has a high affinity for glial cells (Konnerth *et al.* 1985, *Biol. Bull.* **169:** 553), and for neurons. After staining for 1 h in a 0.2 mg/ml solution of RH-155, the extrinsic absorption changes in the molecular layer in response to a single stimulus exhibit a fast component representing the parallel fiber action potential, and a large, long lasting component that reflects the changes in glial membrane potential. This latter signal effectively monitors the glial membranes' response to the accumulation of potassium in the extracellular space. Evidence for this was provided by three kinds of experiments. First, manipulation of the volume of the extracellular space by changing the osmolarity of the medium produced dramatic changes in the size of the glial signal: increasing the extracellular volume reduced the size of the optical change, and *vice versa.* Second, inhibition of the Na/K pump with 10 μ*M* Ouabain, to reduce the rate of potassium clearance from the extracellular space, increased the size and greatly prolonged the recovery of the glial component of the optical signal. Finally, temperature changes

(8°C–19°C) altered the kinetics of the slow component in a manner consistent with their effects on the turnover rate of the pump.

Together with previous findings (Konnerth *et al.* 1985, *Biol. Bull.* **169**: 553) demonstrating the exquisite sensitivity of the slow signal to calcium and calcium blockers, these results suggest that much of the potassium that accumulates in the cerebellum of the skate passes through calcium-dependent potassium channels, and that intracellular calcium is critically involved in its potassium homeostasis.

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Aminoglycoside antibiotics block calcium currents in nerve terminals of the frog neurohypophysis: an optical demonstration using potentiometric probes. T. D. PARSONS, A. L. OBAID, AND B. M. SALZBERG (University of Pennsylvania).

Aminoglycoside antibiotics, including neomycin, streptomycin, and gentamicin, depress evoked transmitter release at the neuromuscular junction by competing with extracellular calcium (Fiekers 1983, *J. Pharmacol. Exp. Ther.* **225**: 487–495). These agents also reduce the size of a light scattering change, correlated with secretion, exhibited by the terminals of the mouse neurohypophysis (Parsons *et al.* 1985, *Biophys. J.* **47**: 447a). We have used the voltage sensitive merocyanine-rhodanine dye NK 2761, and a system for multiple site optical recording of transmembrane voltage (MSORTV) to monitor the action potential in a population of synchronously active nerve terminals in the neurohypophysis of the frog, *Xenopus laevis* (Salzberg *et al.* 1983, *Nature* **306**: 36–40).

Neomycin, at a concentration of 220 μM, reduced the height of the upstroke of the nerve terminal action potential, increased the spike duration, and abolished a prominent after-hyperpolarization resulting from a calcium-mediated potassium conductance. These effects were reversible upon washing, and could be antagonized by raising extracellular calcium from 2 mM to 5 mM.

A pure calcium action potential can be elicited in these terminals by direct field stimulation in the presence of tetrodotoxin (1 μM) and tetraethylammonium (1 mM) (Obaid *et al.* 1985, *J. Gen. Physiol.* **85**: 481–489). Gentamicin, as commonly used in tissue culture media (100 μg/ml, equivalent to 190 μM) decreased the height of the regenerative calcium response, and practically eliminated the after-hyperpolarization. These responses also were observed with neomycin, depended upon dose, and could be reversed by washing or increasing extracellular calcium.

These observations are all consistent with the hypothesis that aminoglycoside antibiotics antagonize the entry of calcium into vertebrate nerve terminals during excitation.

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Subcellular localization of calcium release by light and inositol 1,4,5 trisphosphate in Limulus *ventral photoreceptors.* RICHARD PAYNE AND ALAN FEIN (Marine Biological Laboratory).

The intracellular messenger, inositol 1,4,5 trisphosphate ($InsP^3$), releases calcium from endoplasmic reticulum (ER). The subcellular distribution of $InsP_3$-sensitive ER is central to the messenger role of $InsP_3$ but its location and specialized form within living cells are unknown. We have investigated the distribution and identity of $InsP_3$- and light-sensitive calcium stores in living *Limulus* ventral photoreceptor cells, where light and $InsP_3$ raise intracellular calcium (Ca_i). We injected ventral photoreceptor cells with the photoprotein aequorin and viewed its luminescence with an image-intensifier. $InsP_3$ only elicited detectable aequorin luminescence when injected into the light-sensitive, rhabdomeral (R)-lobe where aequorin luminescence induced by light was also confined. Calcium stores released by light and $InsP_3$ therefore are localized to the R-lobe. Within the R-lobe, $InsP_3$-induced aequorin luminescence was further confined around the injection site, due to rapid dilution and/or degradation of injected $InsP_3$. Prominent cisternae of smooth ER are uniquely localized within the cell beneath the microvillar surface of the R-lobe (Calman and Chamberlain 1982, *J. Gen. Physiol.* **80**: 839–862). These cisternae are the probable site of $InsP_3$ action.

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Synaptic contacts in the outer plexiform layer of the skate retina. HIROKO M. SAKAI (National Institute for Basic Biology, Okazaki, Japan), KEN-ICHI NAKA, RICHARD L. CHAPPELL, AND HARRIS RIPPS.

In the retinas of animals with mixed rod-cone populations, the neural network is organized to segregate (and process) the signals from the two classes of photoreceptor. The neuronal architecture reflects this functional diversity; there are various classes of second- and third-order neurons, with different synaptic connections and appropriate membrane specializations.

The retina of the skate (*Raja erinacea* or *R. oscellata*) contains only rod photoreceptors, and it may be expected to exhibit a less complex synaptic organization. However, our EM study of the structural features of the outer plexiform layer (OPL), where rods make synaptic contact with processes of horizontal and bipolar cells, suggests that this is not the case. The terminal ending of the rod is located in close proximity to the cell's nucleus, giving the appearance that the dense collection of synaptic vesicles and multiple synaptic ribbons are associated with the cell perikaryon. Broad, elongated telodendria issue from the proximal region of the terminal, and extend either laterally or deeper into the OPL to contact the processes of other cells. The single, large invaginated region at the base of the receptor terminal receives processes from horizontal cells that form a dyad in association with each of 2 to 4 synaptic ribbons. Tracer studies of HRP-impregnated cells indicate that these processes usually arise from two or more horizontal cells. Moreover, there appears to be two morphologically distinct types of horizontal cell: one that is cuboidal and located at the distal margin of the inner nuclear layer; another that is a slender elongated element situated just proximally to the distal horizontal cell. Both types of cells produce hyperpolarizing responses to light, and both contribute processes to form the lateral elements of the receptor dyad.

The dendritic processes of bipolar cells also invaginate the receptor terminal, but they do not terminate near the arcuate density of the synaptic ribbon. Instead, membrane specializations are seen between the bipolar cell processes and regions of receptor membrane removed from the ribbon; in some sections, these membrane specializations were associated with aggregates of synaptic vesicles along the receptor membrane.

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Ultrastructure of vestibulo-oculomotor connections in goldfish and winter flounder. R. F. Spencer, M. Weiser, W. Graf, and R. Baker (Marine Biological Laboratory).

Direct demonstration of inhibition in the form of postsynaptic conductances in the vestibulo-oculomotor system of teleost fishes has been elusive, in spite of supportive evidence available from single cell morphological (Graf and Baker 1985, *J. Neurophysiol.* **54:** 900–916) and immunocytochemical data (GABA-ergic synapses) (Graf *et al.* 1985, *Biol. Bull.* **169**: 551). The present study provides further corroborative evidence for inhibition between second-order vestibular neurons and oculomotor neurons. Electron microscopy of the goldfish (*Carassius auratus*) oculomotor nucleus showed axosomatic and axodendritic synaptic endings containing spheroidal synaptic vesicles that established both chemical (presumed excitatory) synaptic contacts characterized by asymmetrical pre-/postsynaptic membrane specializations. In addition, many axosomatic synapses also showed gap junction contacts consistent with electrotonic coupling. Other axosomatic synaptic endings contained pleiomorphic synaptic vesicles establishing chemical synaptic contacts on motoneurons. These presumed inhibitory endings never established gap junctions. Reconstruction of winter flounder (*Pseudopleuronectes americanus*) second-order vestibular neuron synaptic endings stained by intracellular injection of horseradish peroxidase (HRP) showed some axosomatic synaptic contacts of both chemical and electrical nature, and axodendritic endings including those on spines that established only chemical contact zones. Some axosomatic synaptic endings contained pleiomorphic vesicles and established single chemical (presumed inhibitory) synaptic contacts. In comparison with fishes, electron microscopic reconstruction of terminal arborization of excititory second-order vestibular axons in the cat oculomotor nucleus showed only chemical synaptic contacts with dendrites, including spines, of oculomotor motoneurons. Synaptic endings derived from inhibitory vestibular neurons were located on somata or proximal dendrites establishing only chemical contacts.

In conclusion, our data provide ultrastructural evidence for inhibition in teleosts. Furthermore, the collective data point to a common organizational pattern of vestibulo-oculomotor connectivity across vertebrate species with excitatory synapses located predominantly on dendrites and inhibitory synapses predominantly located on somata and proximal dendrites.

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Toadfish sonic motor system. III. Localization of the command nucleus. M. Weiser, M. V. L. Bennett, and R. Baker (Marine Biological Laboratory).

The sonic motoneurons of toadfish comprise a spindle-shaped nucleus in the midline of the rostral spinal cord. Their synchronous discharge at 100–200/s determines the fundamental frequency of the sound generated by contractions of the sonic muscle which is wrapped around the swim bladder. Intracellular recordings from the motoneurons showed that their spikes are driven by synchronous EPSPs separated by IPSPs. Presynaptic fibers also were recorded from and identified by their generating impulses slightly preceding each motoneuron spike. Their activity showed a range of responses; at one extreme impulses could arise from a level baseline or small hyperpolarization, and were evidently recorded close to the motoneurons to which they are electrotonically coupled; at the other extreme impulses were initiated by obvious EPSPs and separated by evident pacemaker activity. The latter responses suggest that these neurons determine the frequency of firing and constitute the command nucleus. Horseradish peroxidase injection of the fibers showed that their cell bodies lie in a horseshoe shaped nucleus around the caudal end of and slightly ventral to the sonic motor nucleus. Sonic discharges could be evoked at low threshold by trains of stimuli at specific sites in the medulla, and essentially identical responses could be evoked by stimulation of the caudal spinal cord. Following complete section through the posterior medulla just rostral to the sonic nucleus, caudal stimulation continued to evoke normal sonic discharges, while responses to rostral stimulation were, of course, blocked. Recordings from sonic motoneurons and presynaptic fibers following the section had the same characteristics as those in intact preparations. These results confirm localization of the command nucleus to the region of the motor nucleus. It is likely that *in vitro* preparations from this region could be used to study ionic mechanisms responsible for initiation, maintenance, and termination of sonic discharges.

Optical measurement of neuron activity during the gill withdrawal reflex in Aplysia.

D. P. Zecevic, J. A. London, L. B. Cohen, and D. Schiminovich (Department of Physiology, Yale University School of Medicine).

Optical measurements were used to obtain an overview of the number and activity of neurons in the *Aplysia* abdominal ganglion during the gill withdrawal reflex. The isolated siphon preparation described by Kupfermann *et al.* (1974, *J. Neurophysiol.* **37**: 996–1019) was used. The siphon was stimulated with a hand-held nylon filament. Action potential activity in cell bodies was monitored via a 124 element photodiode array using absorption measurements on ganglia stained with the voltage sensitive oxonol dye NK3041 (nee RH155).

In a control trial activity occurred in 70 neurons. Over 85% of these neurons had stimulus related activity. When the preparation was habituated, only 50 active neurons were detected. Apparently the firing frequency was reduced in some neurons. We measured activity in 90 different neurons following sensitizing stimuli to a cerebro-abdominal connective.

We compared the optical recordings from cell bodies with extracellular recordings made from the three nerves and two connectives. In about 10% of the cells, activity in a nerve either consistently preceded or followed activity in the cell body with delays of less than 5 ms. In this way we tentatively identify cells as sensory or motor.

We do not know how complete our optical recording was. Earlier experiments on *Navanax* buccal ganglia suggested that the recording was 70% complete. However, because the *Aplysia* ganglion scatters more light, and was larger than the microscope field of view, it is likely that the percent completeness was substantially less than 70%. Using a percent completeness of 50% would mean that there were approximately 200 neurons active in the sensitized preparation. Thus it appears likely that the gill withdrawal reflex has a relatively complex neuronal substrate.

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Pigments and swelling in the lenses of marine vertebrates.

Seymour Zigman, Teresa Paxhia, and William Waldron (University of Rochester School of Medicine & Dentistry, Rochester, New York 14642).

The presence and absence of near-UV absorbing lens pigments and the induction of the lenses of marine vertebrates to swell due to near-UV exposure was investigated. Water-insoluble low molecular weight (dialyzable) pigments that appear to be kynurenines are present in the lenses of sea robins, scup, cod, and some other teleosts, but not in lenses of elasmobranchs (dogfish) or those of at least three teleosts: toadfish, tautog, or sole. Generally, lenses of shallower swimmers contain these pigments, while those of deep swimmers do not. The primary enzyme that oxidizes tryptophan to kynurenines [tryptophan pyrrolase (TP)] is active only in lenses that contain these near-UV absorbing pigments. It appears that lens TP activity depends on activators not present in all species. This may be related to their presence in sunlit environments.

In another study, *in vitro* swelling of the lenses of marine vertebrates in iso-osmotic Ringer's solutions could not be induced readily by exposure to near-UV radiation as occurs in mammalian lenses. This swelling accompanies the near-UV inhibition of Na/K ATPase activity. The relatively high activity of Na/K ATPase in dogfish lens was inhibited but swelling was not induced by near-UV exposure. Tryptophan at 0.02 M in the medium sensitizes Na/K ATPase inhibition by UV. Lack of swelling in marine vertebrate lenses indicates a great resistance by lens fiber cells to lose structural integrity despite losses of Na/K ATPase activity.

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PHYSIOLOGY

Limulus *alpha-2-macroglobulin: first evidence in an invertebrate for a protein containing an internal thiol ester bond.* PETER B. ARMSTRONG (Department of Zoology, University of California, Davis, California 95616) AND JAMES P. QUIGLEY.

The taxonomy of the plasma proteins has been simplified by the realization that each of the several hundred proteins present in the plasma of vertebrates can be assigned to one of approximately 20 classes. One of these is the thiol ester class, which includes alpha-2-macroglobulin (α_2M) and the complement proteins C3 and C4. Members of this class are characterized by the presence of an internal thiol ester bond that is essential for normal function. We report here the first evidence for a thiol ester protein in an invertebrate, the α_2M homologue present in the plasma of *Limulus polyphemus* (Quigley and Armstrong 1985, *J. Biol. Chem.* **260:** 12,715–12,719). Our evidence is of three kinds: (1) functional inactivation by low molecular weight amines, (2) the generation of specific peptide fragments by mild heat denaturation, and (3) the appearance of new sulfhydryl groups during reaction with trypsin. The low molecular weight amine, methylamine, inactivates the ability of *Limulus* α_2M to block hydrolysis of [^{14}C]casein by trypsin and also the ability of *Limulus* α_2M to protect the active site of trypsin from the macromolecular active site inhibitor, soybean trypsin inhibitor. Incubation of *Limulus* α_2M at 80°C for 2 hours generates peptide fragments of 125 kD and 55 kD, comparable to the 125 kD and 67 kD fragments generated by similar treatment of human α_2M. Heat fragmentation does not occur in human or *Limulus* α_2M pretreated with methylamine. The sulfhydryl groups generated during reaction with trypsin have been quantitated with 4,4′-dithiodipyridine. A purified preparation of *Limulus* α_2M capable of binding 4.0 p moles of trypsin/ml generated 10.5 p moles of sulfhydryl groups/ml during reaction with trypsin. The ratio: moles trypsin bound:moles sulfhydryls was 1:2.60 for *Limulus* α_2M and was 1:2.04 for human α_2M.

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Mussel adhesive protein from Mytilus edulis: *applications in orthopaedic research and surgery.* JOHN P. FULKERSON, M.D., AND J. MATHIEU MASSICOTTE (University of Connecticut School of Medicine, Farmington, Connecticut).

Mussel adhesive protein (MAP) obtained from the blue mussel, *Mytilus edulis,* has unique properties, including adhesion in a saline environment, which make it well-suited to surgical and dental applications. This study explores the application of MAP to surfaces relevant to orthopaedic surgeons, and its ability to enhance adhesion of cartilage and bone cells.

Articular and epiphyseal chondrocytes were obtained from 16–20 day chick embryos. Cells ($2–5 \times 10^5$) were released by collagenase and hyaluronidase and then placed in known concentrations over petri plates (35 mm), human bone (1 cm^2), or vitallium discs (2.8 cm^2), with and without MAP (35–140 mg). Cell counts after a 15 minute incubation provided the percent of cells attaching. Scanning electron microscopy was used to examine the treated surfaces.

Fifty-nine percent of epiphyseal chondrocytes attached to vitallium (a metal used for joint replacement prostheses) alone (no MAP) within 15 minutes, while 79% of the cells attached when the metal was coated with MAP ($n = 8$). Thirty percent of chondrocytes (articular) attached to petri plates in 15 minutes *verus* 84% when the plate was coated with MAP. There was no significant difference in the rates of attachment to human bone with and without MAP. Scanning electron microscopy confirmed the attachment of cells to petri plates and vitallium with evidence of cell enhanced attachment (possibly chondronectin) to both MAP-treated and untreated surfaces.

MAP enhances articular and epiphyseal chondrocyte attachment to vitallium and petri plates. These observations may be significant in tissue culture of cartilage and epiphyseal cells, and may have implications in the attachment of appropriate cells to metal in total joint replacement surgery.

This research has been funded by the Arthroscopy Association of North America and the University of Connecticut Research Foundation.

Electron spin resonance studies of the differences in oxidoreductase properties of normal and transformed cells. PETER GASCOYNE, RONALD PETHIG, JANE MCLAUGHLIN, AND ALBERT SZENT-GYÖRGYI (Marine Biological Laboratory).

Electron spin resonance (ESR) studies from this laboratory (Gascoyne *et al.* 1985, *Biol. Bull.* **169**: 560; Pethig *et al.* 1985, *Proc. Natl. Acad. Sci. USA* **82**: 1439–1444) demonstrated that normal and transformed cells are capable of accelerating the rate of decay of a mixture of ascorbyl and semiquinone free radicals in a manner that parallels the cells' state of transformation. We also have found that this effect is associated with an NAD(P)H-enzyme containing an active sulfhydryl group.

We find that accelerated decay of the radicals occurs through either a two-electron reduction of the quinone or a one-electron reduction of the semiquinone radical, and that the ascorbyl radicals are only weakly modified. We have demonstrated that at least three members of the family of disulfide-oxidoreductases are capable of interacting with the ascorbate + quinone mixture in a way that is consistent with the effects observed in the cell studies. Glutathione reductase (EC 1.6.4.2) in the presence of NADPH and oxidised glutathione can achieve this by the two-electron reduction of the quinone. Dihydrolipoamide dehydrogenase (diaphorase, EC 1.6.4.3), in the presence of NADH and lipoamide, also can reduce directly the quinone, and may in addition be capable of one-electron reduction of the semiquinone radicals. DT-diaphorase (EC 1.6.99.2) in the presence of NADH also modifies the radical decay kinetics by reducing the quinone directly.

We conclude that, compared with normal cells, the transformed cells studied may have possessed either an increased NAD(P)H reducing power, an increased activity of disulfide oxidoreductases, or an increased concentration of disulfide substrates such as glutathione or lipoamide. Future work will investigate these possibilities.

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Electrophoresis measurements of the surface charges of normal and transformed cells. JONATHAN PRICE, RONALD PETHIG, PETER GASCOYNE, JANE MCLAUGHLIN, AND ALBERT SZENT-GYÖRGYI (Marine Biological Laboratory).

Investigations have been made of the changes that occur in the transmembrane potential difference (E_m) and the cell membrane surface charge density when mammalian cells undergo neoplastic transformation. In this collaborative program, Dr. Chiu-Nan Lai, M. D. Anderson Hospital and Tumor Institute, Houston, Texas, has determined relative changes in E_m values using a fluorescent dye technique. For the same cell lines we have determined the surface charge using free flow electrophoresis. Normal rat kidney (NRK) cells and a clone of NRK, designated as 6m2, that exhibited a transformed phenotype at 33°C and a normal phenotype at 39°C, were studied together with a clone of 6m2, designated 54-5A4, which is transformed at both 33°C and 39°C. A surface charge density of $-1.42\ \mu C/cm^2$ was obtained for the NRK and normal 6m2 cells at 39°C, whereas at 33°C values of -1.85 and $-1.78\ \mu C/cm^2$ were determined for the transformed 6m2 and 54-5A4 cells, respectively. Seventy-two percent of the extra charge that appeared on the transformed 6m2 cells compared with the normal 6m2 cells was found to be RNase-susceptible. The time-dependent decrease in surface charge that accompanied the shift of the 6m2 cells from their transformed to normal state was found to mirror the increase observed in E_m, and also was comparable to the changes in their morphology and virally coded protein content. Two clones of Friend erythro-leukemic cells, designated DS19 and R1, were also studied. DS19 cells grown in the presence of DMSO exhibited a normal phenotype and had a surface charge ($0.81\ \mu C/cm^2$) 10% less than that of the non-differentiated phenotype grown in the absence of DMSO. Clone R1 exhibited no change in either phenotype or surface charge when grown in the presence or absence of DMSO.

This work was made possible by the Alix Robinson Grant for Bioelectrical Research.

Activation of Limulus *blood coagulation in the absence of detectable endotoxin.* FREDERICK R. RICKLES (Department of Medicine, University of Connecticut Health Center and VA Medical Center, Newington, Connecticut 06111), TOD V. ALBERGHINI, AND PETER B. ARMSTRONG.

Activation of blood coagulation is a fundamental host response to trauma and is utilized to delimit the inflammatory response to pathogens and other noxious stimuli. Previous work on the coagulation reactions of *Limulus polyphemus,* the most completely studied of all invertebrates, suggested that initial activation of a 135–150 kD proclotting enzyme by endotoxin was obligatory for normal coagulation. All previous studies of *Limulus* clotting proteins have utilized material prepared by distilled water lysis of blood cells (amebocytes) in the presence of the inhibitor n-ethylmaleimide (NEM). We report here the first biochemical evidence for an endotoxin-independent coagulation sequence in *Limulus,* the terminal components of which appear identical to those of the classical, endotoxin-mediated sequence described by others. Blood was collected without inhibitors directly into heat-treated, endotoxin-free dishes or washed, granulated amebocytes were prepared and solubilized immediately. Following adherence of the unwashed, live cells, spontaneous degranulation occurred and, subsequently, gel or clot formation was observed. This clot and the residual clot supernate were recovered from the dishes, solubilized, and the proteins in the three fractions analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis. Contaminating endotoxin was excluded by testing all of the materials with a *Limulus* Amebocyte Lysate (LAL) sensitive to 10 pg/ml of endotoxin. A protein with the appropriate molecular weight of the proclotting enzyme (~135 kD) was observed in extracts of either intact amebocytes or of the clot but not in extracts of the clot supernate. A 75 kD protein, presumed to be the clotting enzyme, was observed in both the clot and the clot supernate but was not seen in extracts of intact amebocytes. The 23.5 kD coagulogen, or clottable protein, was visualized in extracts of all 3 fractions but the 17.5 kD coagulin, characteristic of endotoxin-mediated clotting, was observed principally in the extracts of the gel or clot. We interpret this data as preliminary evidence for an endotoxin-independent coagulation system in *Limulus,* perhaps mediated by an NEM-sensitive protease not active in traditional preparations of LAL.

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Muscle function in carp during swimming. Lawrence C. Rome (University of Tennessee), R. McNeill Alexander, Roel Funke, Gordon Lutz, and Marvin Freadman.

Although much is known about the contractile properties of different muscle fiber types, little is known about how they function during locomotion. It is generally assumed that slow fiber types are more efficient than fast ones at slow speeds of locomotion, but they cannot shorten fast enough to enable the animal to move at high speeds. Fish provide an excellent model to test this hypothesis because of the discrete recruitment of the different muscle fiber types (Rome *et al.* 1985, *Science*).

We used two different methods to determine the velocity (V) at which the red (slow) muscle fibers shorten during swimming. Both methods involved high speed motion pictures (200 frames/s) of carp swimming in a flume at speeds from 20 to 60 cm/s at 15°C. In the first, fish were sacrificed and fixed in the body positions observed in the film. Red fibers were teased out and the sarcomere length measured by light microscopy and optical diffraction. In the second, the muscle fiber length was calculated from the curvature of the fish's body.

Preliminary analysis suggests that during normal swimming, sarcomere length undergoes excursions of ± 0.15 μm around a central value of 1.90 μm. This would suggest that fibers are operating on the ascending limb of the force-sarcomere length curve. At 50 cm/s, a speed at which the white (fast) muscle is recruited in addition to the red, the V of the red muscle was calculated to be 2.5 muscle lengths/s. This value exceeds that measured for the maximum speed of shortening (V_{max}) of red fibers (Johnston *et al.* 1985, *Exp. Biol.*). This would suggest that the fast fibers force the slow fibers to shorten at speeds greater than their V_{max}, and thus the slow fibers cannot contribute to force generation at high swim speeds. This conclusion is tentative, however, because Johnston *et al.* used much larger carp which would probably have slower V_{max}'s than ours. We will measure V_{max} of muscle fibers from our smaller carp to further test this conclusion.

Supported by an MBL Summer Fellowship (L.C.R.), by University at Tennessee Faculty Development and Research Inventive Awards (L.C.R.), and by NSF Grant # OCE-8416257 (M.A.F.).

Stimulus-response coupling in marine sponge aggregation: metabolic and functional studies with arachidonic (20:4) and docosahexaenoic (22:6) acids. Moseley Waite, Sarah A. Davidson, William J. Riesen, and Gerald Weissmann (Marine Biological Laboratory).

Dissociated cells (Ca^{++}-free seawater) of *Microciona prolifera* aggregate rapidly when exposed to calcium and ionophores, or to ionophores and phorbol esters. Secreting an aggregation factor during stimulus-response coupling, the cells fuse secretion granules with the plasmalemma. We now show that the cells undergo profound changes in lipid metabolism. Lipid analyses were performed by thin-layer, gas-liquid, and high-performance liquid chromatography with selected compounds subject to UV and mass spectrometry. Sponge cells had 1.1 μmole of lipid P, and 1.8 μmole fatty acid/10^8 cells; the major phospholipids were: phosphatidylcholine 40.5%, phosphatidyl serine/inositol 31.4%, and cardiolipin-like material 28.1%; phosphatidate was also identified. The major (20%) unsaturated fatty acid was docosahexaenoic (22:6); the major saturated fatty acid was palmitic (16:0); arachidonic acid (20:4) comprised 2%. Both docosahexaenoic and arachidonic acid (>10 μ*M*) had profound effects on sponge cell function, enhancing aggregation elicited by calcium and ionophore and overcoming its inhibition by colchicine and vinblastine. Colchicine inhibition also was relieved by phorbol esters (>50 n*M*) but not phorbol, and by diglyceride, suggesting that 22:6 and 20:4 might influence protein kinase C. The effects of 22:6 and phorbol ester in turn were abolished by calmidazolium, suggesting that intracellular calcium transduced their action. Aggregation was accompanied by the preferential incorporation of 22:6 and 20:4 into diglycerides and phospholipids as opposed to triglycerides in resting cells. The data show that these ancient multicellular creatures remodel their phospholipids during stimulus-response coupling and support the hypothesis that the twin signals of protein kinase C and intracellular calcium are influenced by products of lipid remodelling (*e.g.*, 22:6, 20:4, diglyceride).

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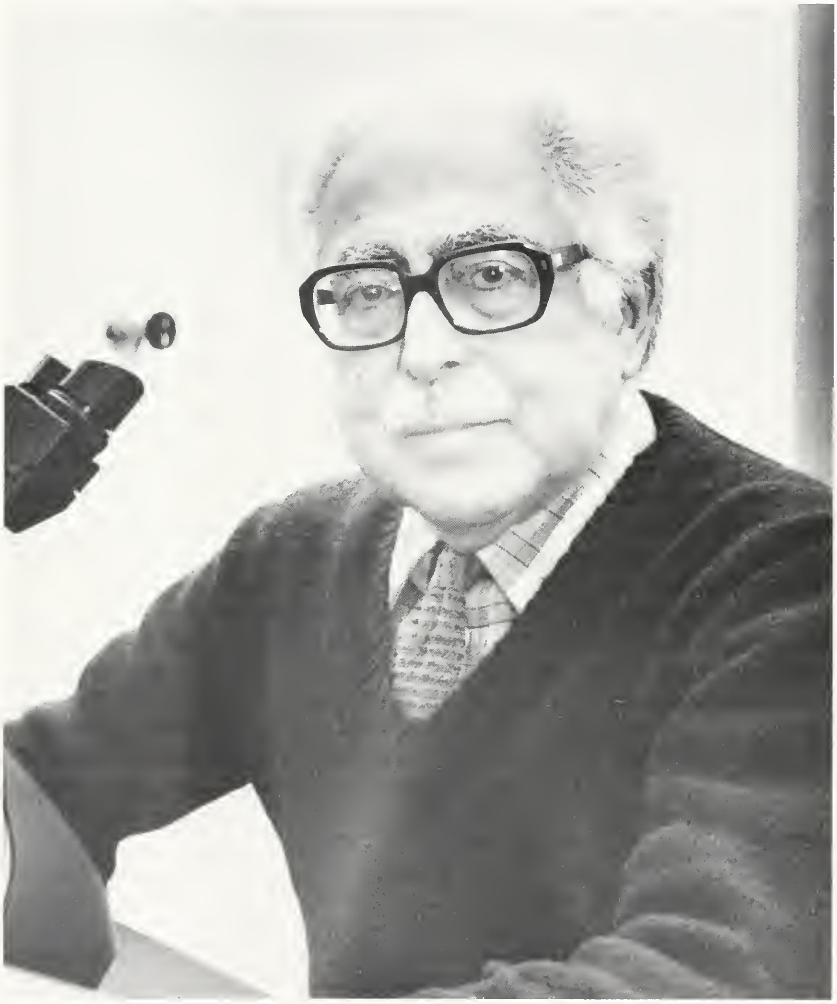

Alberto Monroy (1913–1986)

Reference: *Biol. Bull.* **171**: 507–508. (December, 1986)

ALBERTO MONROY

(1913–1986)

In high green summer at Woods Hole, on a weekend of science and civilized hospitality, death paid its call upon Alberto Monroy. He had returned joyously to this shore, his troubled health having improved sufficiently to permit it. Death came as it usually does: rudely, barging in without so much as a knock at the door, heedless of other plans the unprepared host had made.

This issue of *The Biological Bulletin* is dedicated to Alberto Monroy. It is not a *Festschrift*. It prints the last paper upon which he had been engaged before his busy hand was stayed. There will be proper *Festschriften:* Alberto Monroy was a member of many Academies and distinguished scholarly societies. Proper scientific biographies will be prepared, and well worth the preparation. His career as an embryologist spanned the biological revolutions of this century following upon the dual triumphs, in the 1930s, of metabolism and genetics. What I write here is not the analytical evaluation that needs more distance from the man than I can step off.

Neither, however, is it merely an obituary. He was too good a friend to for that, and too important a member of this, the community of the Marine Biological Laboratory, to deserve it of us. A bald obituary, sparing of adjectives, does have dignity; but a dedication for Alberto Monroy, made so soon after his loss, must convey something, however briefly, of the nature of that loss.

Born to a princely family in Palermo, Italy, in 1913, Alberto Monroy gave early evidence of intellectual gifts. He studied medicine at the University of Palermo and received the M.D. in 1937. Enthusiasm for biological research sent him quickly into the academic world: he was soon an Assistant Professor of Anatomy at Palermo, and establishing what was to be a broad range of research interests. After World War II he went to Naples to head the physiological laboratory of the Stazione Zoologica; he returned to Palermo as Professor of Comparative Anatomy in 1952.

There he would lead, until 1969, the productive Institute of Comparative Anatomy, and from that seat, influence personally and positively the whole course of Italian biology. In modest but efficient quarters, on the narrow Via Archirafi in a less than opulent part of town, Monroy's institute had a more glittering and international clientele than the royal hotel at the other end of the city, or the elegant spas of Mondello.

With great generosity of spirit, despite his strong ties to Sicily, he met the need of Italian biology for his combination of administrative skill and competence in modern biology by founding and directing the Laboratory of Molecular Embryology at Naples. Then, in 1976, he agreed to serve as Director of the Stazione Zoologica.

In 1982, aged 69, but intellectually a man very much in his prime, he resigned the post and became Head of the Stazione's Laboratory of Cellular and Developmental Biology. The change was nothing like a retirement. The productivity of Monroy and his always-abundant, talented co-workers (among the first of whom had been his wife, Anna Monroy-Oddo) rose to a level surpassing even that of the busy years in Palermo.

For his energy, his unselfish administrative services, and his undiminished output of quality research he was famous and honored, although not, perhaps, sufficiently so.

He was a foreign honorary member of the American Academy of Arts and Sciences, recipient of an honorary doctorate from the University of Chicago, winner of the Albert Brachet Prize of the Belgian Royal Academy of Sciences. He was a respected visiting professor at important institutions around the world: the University of Chicago, Rockefeller University, the California Institute of Technology, the University of Puerto Rico, the University of Nagoya. He was an F. R. Lillie Fellow at the MBL in Woods Hole, a key member of its Fertilization and Gamete Physiology program, and among the most respected of its Trustees. The universal trust invested in him is evidenced by posts of leadership he held in such international bodies as Euratom, EMBO, and the International Society of Developmental Biologists.

His last two years were plagued by ill health; yet they brought a renaissance of his broadest interests in development. His research publications alone, *i.e.*, excluding books written and edited, covered comparative fertilization, the evolution of germline segregation, identification of a fucosyl glycoprotein sperm receptor in the vitelline coat of *Ciona intestinalis,* the thermodynamics of sperm-egg interaction, differentiation of the vitelline envelope in *Xenopus* oocytes, and essays on an important period in the history of embryology. Two days before his death he urged me to join him in a new project, recalling those happy times when my laboratory at MIT and his at Palermo were joined in a cooperative program. It was an exchange from which our students and colleagues, and, if I may say so, the field of molecular embryology, derived much benefit.

But all this is too pale a representation. It says nothing of the unique personality to which the citations and the recognitions were merely an adornment. Standing at his sad bedside, observing the body from which ebullient life had so recently fled, I thought not of his biology, but of his unfailing good cheer; of how, in him, optimism had a rare coexistence with intelligence. I thought of how much we had loved him, and of his ties to so many of us. He was an unsurpassable model for administrators. Against the norm for these, our learned professions, he had genuine delight in the achievements of others, even when they were—by their admission—his rivals. He was everywhere at ease: by linguistic skill and a transparent honesty he could live as a true cosmopolitan. The Monroy home stood for the best of Italian culture: perfect taste, consideration, flexibility, a warm generosity.

My daughter Aline, as a child, thought him handsome: my daughter Wendy judged him clever; to my wife, he was the ideal of a gentleman, brought to life in this ungentle century. In fact he was a happy innocent: by virtue of innocence combined with a keen mind, great energy, and an impregnable private happiness, he almost always had his way. No politician, public or scientific, could resist for long good sense clothed in Alberto Monroy, and no rival could deny him the return of friendship with which he was himself so free.

Death does have its sting: there is no denying it. But life can, for the lucky, have its joy. Alberto Monroy got a good share of it, enlivened by the contemporary adventure of developmental biology. We all participate in it, but he gained more and could give more than did most of his contemporaries. *Ave,* Alberto, *atque vale.*

Paul R. Gross
Woods Hole, September, 1986

Reference: *Biol. Bull.* **171**: 509–519. (December, 1986)

A CENTENNIAL DEBT OF DEVELOPMENTAL BIOLOGY TO THE SEA URCHIN[1]

ALBERTO MONROY

Zoological Station, 80121 Naples, Italy

> Most scientific problems are far better understood by studying their history than their logic.
>
> E. Mayr—*The Growth of Biological Thought.* Harvard Univ. Press, 1982.

INTRODUCTION

The fortunes of a field in Biology depend to a large extent on the organism selected for investigation. A successful case in point is the sea urchin in the study of development. The other side of the coin is that once such an organism has been discovered there is a tendency to consider it *a general model* for all organisms. Again, this has been the case of the sea urchin embryo.

There is no question that the sea urchin embryo has played a key role in the field of embryology, equaled only by the amphibian embryo. Hence it seemed to me worthwhile to take the opportunity of this occasion to trace the early history of why and how the sea urchin came to acquire such a position. And in particular to analyze to what extent work on the sea urchin has contributed to laying the foundations of Cell and Developmental Biology, not only through important discoveries but also through the formulation of problems that have become central to present day Developmental Biology.

Accordingly I shall confine myself to the period that spans from the 1870s to the first decade of our century. Even granted this limitation, the topic is too vast for this presentation, and hence I shall discuss only the three topics in which experiments with the sea urchin egg and embryo have either played a major role or have contributed significantly to the understanding of basic events of development. The three topics are: first, fertilization and initiation of development; second, the debate on the impact of evolution on the events of development; and finally, nuclear control of the events of development.

When and why did the sea urchin start attracting the attention of zoologists interested in development? The earliest work on sea urchin development that I have been able to trace is that of Dufossé (1847). Even though his illustrations were rather crude, they nevertheless showed how well suited this embryo was for the study of development, its main advantages being its transparency and the ease with which it could be raised in the laboratory. Indeed, what zoologists were primarily aiming at, now that good quality microscopes were available, was to "see" what takes place within an egg while it develops; in this respect the sea urchin embryo was almost unique.

FERTILIZATION

The two major discoveries in the field of fertilization were made thanks to work on the sea urchin egg. The first was Hertwig's discovery (1876) that the key event of

Received 11 July 1986; accepted 21 August 1986. This article was received and accepted shortly before Dr. Monroy's untimely death on 23 August 1986.

[1] Introductory lecture delivered at the MBL on 25 August 1985 on the occasion of the Symposium on Sea Urchin Development.

fertilization is the fusion of the nucleus of the spermatozoon with the nucleus of the oocyte; the second was the discovery of artificial parthenogenesis, a quarter of a century later, by Loeb (1899).

Indeed, in spite of the fact that there was an almost general consensus that the initiation of development required an interaction between the spermatozoon and the egg, how this occurred and what it was leading to remained a mystery. For example, Bischoff (1847) asserted that "the semen acted by contact . . ."

Hertwig's discovery that the zygote nucleus is the product of the fusion of the nucleus of the egg with *one* sperm nucleus, led him to postulate that "the most important event (of fertilization) is therefore the fusion of the two cell nuclei." Hertwig's discovery is a milestone in the history of development as it was the first and indeed the *sine qua non* step to understanding the role of the two parents in the development of the new organism. However, once the two nuclei have fused, what processes trigger the egg into division? Boveri (1888, 1895a) and Wilson and Mathews (1895) discovered that the sea urchin egg lacks a centrosome and hence it lacks the ability to engage in cell division: it is the "injection" of the centrosome of the spermatozoon that awakens the egg from its dormancy: this is known as the Boveri-Wilson theory of egg activation. Wilson and Mathews (1895) also showed that the centrosome in the mid piece of the spermatozoon is the site from which the sperm aster arises, and they suggested that the sperm aster is somehow involved in the movements of the sperm nucleus within the egg. It must be noted that the mechanisms by which the centrosome organizes the sperm aster and triggers the movement of the egg nucleus are still largely unknown. As has been aptly pointed out by Mazia (1984) "The trouble was and is the difficulty of seeing the centrosome." As noted by Boveri (1901) this makes the sea urchin egg one of the least appropriate materials for the *in vivo* study of the centrosome.

Nevertheless, the "mystery of the centrosome" excited the curiosity of biologists and they resorted to a number of model experiments in the attempt to penetrate the mechanisms by which the centrosome organizes the mitotic spindle. One such experiment was that devised by Giardina (1902) to simulate the mechanics of the movement of the sperm nucleus and its fusion with the egg nucleus in the sea urchin egg fertilization. He floated a small boat-shaped wax plate that simulated the sperm head, on water. He placed a droplet of alcohol in a recess on the rear end of the plate and the diffusion of the alcohol caused the wax plate to advance rapidly. From this experiment he suggested that the centrosome was the site of diffusion of tensioactive substances that caused the movement of sperm nucleus. Upon observing the events of the fusion of the two pronuclei he also noticed that the egg nucleus started moving toward the sperm nucleus upon being reached by the radiations of the sperm aster and in doing so it acquired an ameboid shape. This he attributed to substances that diffuse along the radiations of the sperm aster and that lower the surface tension of the nuclear envelope. These observations, however naïve, are, to my knowledge, the first attempt to offer a physico-chemical interpretation of the mechanics of the encounter and fusion of the two pronuclei in fertilization.

I should also like to rescue from oblivion the important observation made by Meves (1912) that the sperm mitochondria do not replicate in the egg cytoplasm. Meves noted that in the sea urchin, the sperm mitochondrion, though entering the egg, remains a compact body that at the first cleavage passes into one of the two blastomeres and soon fades away. As far as I am aware, this is the first suggestion that the mitochondria of the embryo are of maternal origin.

At the turn of the century artificial parthenogenesis was the epoch-making discovery in the field of development (Loeb, 1899). The discovery stirred great interest and heated discussions not least because of its theoretical and religious implications. The discovery arose from a chance observation during experiments Loeb was conducting to test his working hypothesis that ". . . changes in the state of matter . . . might play an important role in the mechanics of life phenomena" (1899). While studying the effect of ions on the sea urchin egg he discovered that treatment of unfertilized eggs with a hypertonic solution of $MgCl_2$ resulted, upon their return to seawater, in the elevation of the fertilization membrane; some of the eggs even developed into plutei. He thus concluded that ". . . the unfertilized egg of the sea urchin contains all the essential elements for the production of a perfect pluteus. . . . All the spermatozoon *needs* to carry into the egg for the process of fertilization are ions to supplement the lack of the one or counteract the effect of the other class of ions in the sea water, or both. The spermatozoon *may* however carry in addition a number of enzymes or other material" (1899). According to Loeb (1913) the process triggering the activation of the egg is, however, a *transient surface cytolysis* brought about by a *lysin* bourne by the spermatozoon: a theory that largely holds to these days.

It was Loeb's discovery that prompted Warburg to use the sea urchin egg for the study of the biochemical processes controlling cell division. Warburg was interested in the problem of how neoplastic cells escape growth control; but he realized that this question would remain unanswered until one understood how the division of normal cells is controlled. The sea urchin egg appeared to be an ideal material to approach the problem. Thanks to Loeb it had indeed become possible to "activate" the eggs by chemical means. In a series of papers published between 1908 and 1910, Warburg analyzed in great detail his original discovery that within a few minutes of fertilization or parthenogenetic activation, the sea urchin egg undergoes a several-fold increase of oxygen consumption. Warburg's papers are a pleasure to read even now. They are a model of scientific ingenuity and rigor, and they lack the verbosity that so often obscures the papers of his contemporaries. As often happens after a breakthrough, these discoveries led to the hasty generalization that the blockage that keeps the unfertilized egg from engaging in cell division lies in an inhibition of the respiratory chain—a generalization that comparative studies soon proved to be incorrect, but which nevertheless gave rise to a number of important investigations.

However, it remained for F. R. Lillie to formulate a theory of fertilization based on the interaction of specific substances (fertilizin and antifertilizin) secreted by the gametes. According to the theory, not only the encounter of the gametes depended upon the interaction, but the actual activation of the egg resulted from it (Lillie, 1919; Monroy, 1965; Manning, 1983). Specifically, Lillie drew attention to the analogy between gamete interaction and antigen-antibody reactions. This provocative idea was taken up and further developed by A. Tyler and his students (see Tyler, 1948; Metz, 1967); indeed, these were among the pioneering investigations in the field of biochemical embryology. However, neither immunology nor biochemistry had yet progressed to the point where they could provide the conceptual and technical tools to approach the problem of fertilization, at least in terms of the interaction between specific substances. For a number of years fertilization was often considered an almost sterile field.

Revival of interest in fertilization came from morphology: namely from the discovery of the acrosome reaction (Dan, 1952) and its role not only in the fusion of the spermatozoon with the egg, but as the key event in gamete interaction and in the activation of the egg (Colwin and Colwin, 1963).

In this connection I should like to mention an essay by Theodor Boveri (1902) that I consider of great importance for the clarity with which the problem of fertilization is formulated. Curiously, this article has fallen into oblivion and is not quoted even in Lillie's "Problems of Fertilization" (1919) in which all Boveri's other papers are mentioned. In this essay Boveri indicated the three main conditions that must be fulfilled to ensure the success of fertilization. First, the gametes must be in a repressed condition, *i.e.,* "they should not be able to develop by themselves, and in fact they should be blocked in such a way that inhibition is released by the other cell." Second, "the two cells must be able to find each other." The third condition is a corollary of what Boveri called the "equivalence of the germ cell," whereby he proposed that "in the same way we say that the spermatozoon fertilizes the egg, so we may say that the egg fertilizes the spermatozoon." This means that in fertilization neither gamete behaves passively in the sense that one is the activator and the other the activated one: *for the success of fertilization the gametes must activate each other.*

I have indulged somewhat in the discussion of fertilization because work on the sea urchin in this field has produced a wealth of discoveries that have general significance in Cell and Developmental Biology. The sea urchin egg has been, and still is, one of the most suitable objects for the study of the processes of fertilization, from the morphological to the molecular level. And even nowadays when other organisms, notably the mammalian egg, have become amenable to experimental analysis, the sea urchin egg has remained a point of reference.

ORGANISMS AS PRODUCTS OF HISTORY

Towards the end of the last century a group of young German embryologists (led by Wilhelm His and Wilhelm Roux) began to show their dissatisfaction with the role assigned by Haeckel (see in particular, Haeckel, 1866) to embryology. Haeckel saw embryology as a mere tool, albeit a most powerful tool, with which to construct animal genealogies: *the embryo per se was uninteresting* (see also Allen, 1985; Monroy and Groeben, 1985). von Baer (1828) had been struck by the close similarity of the early stages of development of vertebrate embryos that sometimes made it difficult to decide to which class they belonged. Haeckel made the great leap and formulated the *general biogenetic law that ontogenesis is an abbreviated recapitulation of phylogenesis.* The law was acclaimed as one of the major conquests in natural history, and the ambition of almost every zoologist was to exploit the information that could be obtained from the study of embryonic development to trace the "Descendenz" of a particular animal group, eventually to discover the original ancestor. This is best expressed by the analogy formulated by Anton Dohrn, the founder of the Naples Zoological Station and a convinced Haeckelian himself, "In the same way as linguistics reconstruct original languages . . . the zoologist should be able to outline a comprehensive picture of an animal group from a large number of embryological data . . . to identify the ancestor of the whole group" (Dohrn, 1872). The view of the new school was just the opposite. To them phylogenetic trees were uninteresting, to say the least, as they lacked any supporting evidence. How, they asked, can genealogies be built with an object, the embryo, about whose mechanisms of development so little is known? Their view was that the primary goal of embryology should be the analysis of the *mechanisms* underlying embryonic development. Hence embryology should become an experimental discipline along the lines of Physics and Chemistry. A new term was coined, "Entwicklungsmechanik," which also embodied the philosophical concept of the mechanistic approach to the study of life.

The new approach reversed the trend of embryological research and *the study of the embryo became interesting "per se."* Hence, *all* stages of development were interesting, and in particular the earlier ones, from fertilization on, rather than only those following gastrulation as maintained by Haeckel (on the assumption that the original, ancestral, form of all animals was a gastrula-like organism, the gastrea, 1866). The study of the early stages of development promised to provide information regarding the *organization* of the egg and in fact about its physiological properties, which, after all, must be the determining elements of development. This problem could be approached only by experimental means: descriptive methods in morphology could not help since the cells looked alike during the early stages of development. When brought to the extreme, as Driesch did (see in particular, 1899) this trend meant not only the rejection of phylogenetic considerations in the study of development, but the rejection of the descriptive approach as a whole.

When Roux reported that killing one of the first two blastomeres of a frog embryo resulted in the surviving blastomere developing into a half embryo (1888), Driesch realized that experiments of this kind could provide important clues as to the organization of the egg. In fact they could answer the question as to whether, as Roux's experiments seemed to suggest, the organization of the egg was already laid down from the time of fertilization at least. However, the experiments he undertook with the sea urchin egg gave a very different kind of answer. Separating the blastomeres surgically was impraticable in those days. [It was not until Sven Hörstadius devised his sophisticated technique (in the early '30s) that operations of great precision could be carried out on the sea urchin embryo.] Driesch, however, found that blastomeres could be separated by vigorously shaking cleaving embryos (1891). Although many blastomeres suffered damage as a result of shaking, some survived and proceeded to develop. Contrary to the results obtained by Roux on the frog embryo, in the sea urchin each of the first two blastomeres developed into a normal-looking, though undersized pluteus. Hence, at least up to a certain stage, the sea urchin embryo behaved as a *harmonic equipotential system*. Driesch, however, was soon able to offer a mechanistic interpretation of these apparently contradictory results. He had previously found (1892) that the cleavage planes were entirely upset in sea urchin embryos compressed between a slide and a coverslip; and in fact in some cases cleavage resulted in the blastomeres lying flat all in the same plane, forming "plates." Nevertheless, upon release of the pressure, the blastomeres were able to reorganize themselves and give rise to a sphere identical to a blastula which eventually developed into a normal larva. In Roux's experiment, on the contrary, the dead blastomeres were not removed making it impossible for the blastomeres "to reorganize a spherical blastula: the blastula was still a half blastula" (1893).

It would be outside the scope of this essay to enter into the details of the experiments that Driesch conducted on the isolated blastomeres. There is, however, an important observation (1898) that is worth mentioning. He had observed that the number of cells of the primary mesechyme is constant and, in fact, typical for each sea urchin species. In embryos originating from the first two separated blastomeres, the number of primary mesenchyme cells was one-half that of the normal embryos. Hence, he concluded, there must be "signals" that stop cell division at precisely specified times. "Why do cell divisions stop? And does the stimulus that stops division come from within or from without the cell?" By asking these questions Driesch put his finger on one of the most challenging problems that still confronts us, *i.e.,* what are the mechanisms whereby the cells are able to count the number of cell divisions in the various cell lines of the embryo?

While going over the literature for the preparation of this essay, I was struck by the fact that whereas at the Naples Zoological Station, most embryologists used the sea urchin embryo for their investigations, such was not the case at the Marine Biological Laboratory in Woods Hole (with the exception of T.H. Morgan). At the MBL, in fact, the sea urchin egg was used almost exclusively for cell physiological studies and for the study of fertilization. It seems to me that this can be explained by comparing the development of embryological research in Europe and in the United States at the turn of the century.

American embryologists acknowledged the importance of the study of the early stages of development and in particular of cleavage. However, largely under the influence of C.O. Whitman, they stressed the importance of detailed knowledge of the *destiny* of the individual blastomeres. Indeed, as stated by E.B. Wilson (1899) ". . . it is in the determination of common origin as well as common fate . . . that the principal significance of recent work on cell lineage seems to me to lie." This analysis could indeed provide important information as to what we would call today the "program of development," which in fact should be traced back to the unfertilized egg.

It is in the work of Whitman on the development of *Clepsine* (1878–1888) that the concept of a program of development is, however vaguely, spelled out. "The egg," Whitman writes, "is the architect of its own destiny . . ." and "Every ontogenic *form* presupposes a preliminary arrangement."

For this kind of analysis the sea urchin embryo was not as suitable an object as the embryo of other marine organisms, such as the mollusks, the anellids, or the tunicates, in which, due to their determinate type of development and to some recognizable markers of the blastomeres, cell lineage could be followed.

A result of this different approach to the study of early development was that the MBL embryologists did not share either Hans Driesch's negative attitude towards descriptive methods or his refusal to take into account the role of evolution in shaping the events of development. The relationship between development and evolution was indeed one of the most serious concerns of the MBL embryologists. [However, it should be mentioned that Driesch's attitude was not shared by all embryologists in Europe. Notably, Boveri stated that "the encounter between the cell theory and the theory of evolution has been one of the greatest contributions to the concept of the *historical character of the organisms*" (1906).]

In two lectures delivered at the MBL, Wilson (1899) and Whitman (1895) stressed that the evolutionary history of organisms cannot be ignored in interpreting the events of development. In Wilson's words "every embryonic stage is the manifestation of the evolutionary history of the organism and an adaptation to its present environment." And then "while it is true that the normal operations of the organism are essentially physiological problems, we must nevertheless not lose sight of the cardinal fact that the organization of the idioplasm that is at the bottom of such operation is an inheritance from the past." And he also did not fail to express his disagreement with Driesch's position in the letters that they exchanged over the years. (Microfilms of the letters of E.B. Wilson to H. Driesch were kindly supplied by the library of the University of Leipzig, DDR, "Nachlass Hans Driesch." Quoted with permission.) Unfortunately, I have not succeeded in tracing Driesch's letters to Wilson.

For example, in a letter of 1 July 1894, Wilson wrote "I still think that you go too far in your condemnation of comparative morphology."

Whitman's attitude was even more explicit as he stated that the organisms are inexplicable without regard to the history of life.

While the recognition of the impact of evolutionary history of organisms on embryonic development was gradually gaining ground, the possibility of its experimental verification appeared remote, if not impossible.

In the Preface to his "Experimental Embryology" (1927), T.H. Morgan wrote, "it seems then more worthwhile to drop the old fruitless discussion on the meaning of development and to turn to the many problems that already presented themselves at almost every stage of the process." It is remarkable that in the whole book the word

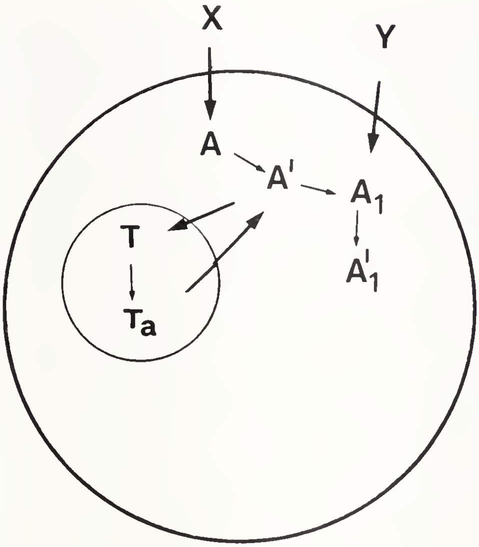

FIGURE 1. Schematic representation of Driesch's (1894) view of the nuclear-cytoplasmic interactions controlling cell differentiation. X is an external stimulus that changes the cytoplasmic component A into A'. This in its turn activates the nuclear component T to T_a which moves out of the nucleus and changes A' into A'_1: this initiates the cascade of events resulting in the differentiation of the cell. The change of A' into A'_1 may be brought about by the external stimulus, Y, that bypasses the nucleus.

"evolution" does not appear even once. As a matter of fact, Morgan was largely responsible for the long divorce between genetics and embryology by emphasizing that evolution was a concern of genetics but had nothing to do with embryology. The impression one gains is that both geneticists and embryologists were almost afraid of risking their reputations by engaging in discussions on the relationships between evolution and development: or at least that any such discussion would be to say the least, fruitless.

THE ROLE OF THE NUCLEUS IN DEVELOPMENT

This is one of the most fascinating chapters in the history of modern Biology and one in which work with the sea urchin embryo has made major contributions.

The problem of the hereditary material which, by the turn of the century, was largely accepted to be contained in the chromatin of the nucleus, was foremost in the mind of most embryologists. Of particular historical interest in this regard is an essay by Driesch (1894) in which he theorized on the polarity of the egg as being due to the distribution of electrical charges of cytoplasmic components; on the role of cell contact and chemically mediated inductions; and in particular on the role of the nucleus in cell differentiation, which he conceived as involving the "activation" of nuclear components though without a permanent change of the overall composition of the nucleus. In Driesch's own words "All the elementary processes are released through stimuli exerted on the nucleus. The nucleus must be set in motion in *such a way that its totality remains unchanged* (my italics). The substances which cause the individual elementary processes do not originate directly in the nucleus, but in the cytoplasm under the direction of the nucleus. The activation of a nuclear substance results in a change in the cytoplasm and this sets in motion a new elementary process" (Fig. 1).

It is remarkable how, even vaguely, the idea of what is now referred to as differential gene activation appeared to Driesch as a prerequisite to explain the control exerted by the nucleus on developmental processes. However, the concept was spelled out most clearly by Morgan (1927) when he asked "How is the ordered sequence of events, that takes place in the cytoplasm, related to the activity of the genes in the chromosomes if there is no correspondence of arrangement in the two? Is it due, for example, to the sequence in which the genes become active?"

The reasoning that started both Driesch and Boveri on their hybridization experiments was that the only vehicle of the paternal characters expressed in the offsprings had to be the sperm nucleus because the spermatozoon essentially lacks cytoplasm. This theory could be tested by experiments of crossing between two species closely related but sufficiently different in some of their characters to be easily recognizable in the offsprings. This, they found, can be achieved in the sea urchin. In particular, clear cut answers should, in principle, be obtained by introducing a spermatozoon of one species into an *enucleated* egg or fragment of another species: the egg fragmentation technique appeared to offer unique opportunities to carry out this experiment. (Years later—1918—Boveri himself discovered that the nucleus of the shaken eggs often breaks up and this gives the false impression of an enucleated egg fragment). Driesch had observed (1898) that the eggs of *Echinus* cleave at a faster rate than those of *Sphaerechinus:* the hybrids *Sphaerechinus* ♀ × *Echinus* ♂ cleave at the rate of the egg. And also the number of the cells of the primary mesenchyme was that of the maternal species. [The results obtained by Boveri (1903) were at variance with the latter point since they indicated that in the hybrids the number of the cells of the primary mesenchyme is intermediate between that of the two parental species.] Hence he concluded that at least as far as these two characters are concerned, the "means" (Mittel) must be located in the cytoplasm of the egg; where, it could not be said. What did appear certain was that the egg nucleus could not be considered the only initiator of development. Boveri pursued the study of hybrids throughout his life. One of his most important discoveries was that in the hybrids the paternal characters begin to be expressed after gastrulation and the characters of both parents are equally represented in the young pluteus. His general conclusion (1903) was that "in general one can say that the purely maternal characters are limited to the earlier stages. The paternal characters show up most distinctly in the young pluteus." Type and rate of cleavage belong to what he called "präformierten Eiplasmaqualitäten."

However, the hybridization experiments could not be properly interpreted until Baltzer discovered (1910) that in some hybrid combinations the paternal chromosomes are eliminated during cleavage; in which case development is arrested at the blastula stage. On the other hand, the compatible hybrids, in which development proceeds beyond gastrulation, have led to a number of important discoveries. Among them, I should like to mention that the hybrid *Paracentrotus* ♀ × *Sphaerechinus* ♂ was used for the first application of immunological techniques to the study of development (Harding *et al.,* 1954): they showed that the expression of a paternal antigen occurs after the mesenchyme blastula stage.

The most important contribution to the "theory of the nucleus" came, however, from Boveri's studies on polyspermic eggs (1907). He showed that in polyspermic eggs the chromosomes are partitioned randomly among the blastomeres. Thus, when the first four blastomeres are taken apart, at variance with normal embryos in which a larva is obtained from each blastomere, in this case some of the blastomeres develop normally, whereas others do not develop at all or become arrested during cleavage. Since he could not detect any correlation between number of chromosomes and developmental ability of the blastomeres, he concluded that what is important for development is not so much *the number* of the chromosomes as *their quality:* hence the chromosomes must be different from one another. And "From this we must conclude that only a precise combination of chromosomes, probably only the totality of those which are contained within each pronucleus, represent the entire nature of the form of the organism . . . (in) the embryonic development of which unfold the qualities of the nuclei".

Thus, at the turn of the century the problem of the role of the nucleus in development had become one of the most intensely studied areas in embryology. Then followed a long period during which embryologists, with some notable exceptions such as C.H. Waddington and J. Brachet, appeared totally to ignore the existence of the nucleus. Concerning the sea urchin, the situation was particularly bleak. Indeed, until a few years ago a severe limitation to the use of the sea urchin embryo for any advanced analysis of development appeared to be its lack of genetic markers and in particular of mutations that affected specific stages of development. This drawback has now been largely overcome by recombinant DNA techniques. As stated by Davidson (1985) . . . "Molecular Biologists are now rapidly providing the conceptual armature erected by Boveri with a body of detailed knowledge."

Indeed, abundance of information on sea urchin development together with that on the evolution of the genome and the wealth of paleontological data (which in the case of the echinoderms are also quite abundant) makes one confident that the sea urchin embryo will continue to play a key role in Cell and Developmental Biology throughout the 21st Century.

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There are two passages in the correspondence between Wilson and Driesch that are worth mentioning in this context. In a letter of 5 June 1893, Wilson writes: "I conceive the ontogeny as a series of actions and interactions between the blastomeres which result in a steady increasingly specializations of the idioplasm, and in which each term conditions the following term." And on 25 November 1895, he clarifies the meaning he attaches to the "*word idioplasm* (by which I meant the specifically organized egg substance, whether nuclear or cytoplasmic)."

Wilson, E. B., and A. P. Mathews. 1895. Maturation, fertilization and polarity in the echinoderm egg. *J. Morphol.* **10:** 319–342.

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DEVELOPMENT OF SPATIAL ORGANIZATION IN PALLEAL BUDS OF THE COMPOUND ASCIDIAN, *SYMPLEGMA REPTANS**

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ABSTRACT

The continuous observation of living animals and the reconstruction of serial sections of fixed specimens have revealed 15 developmental stages and their timetable in palleal buds of the compound ascidian, *Symplegma reptans.* The bud primordium (stage 1) appeared on the right anterior region of the atrial epithelium of its parental bud (stage 9). It evaginated to form a vesicle along with the epidermis (stage 3) about two days later (at 18°C). Stage 4 was characterized by the formation of a test vessel through which the bud received active bloodflow. Body axes and bilateral asymmetry first became visible at this stage. Rudiments of the neural complex, pharynx, gut, and endostyle were formed directly from the inner vesicle of the bud. The neural complex placode was the first organ rudiment observed, in 3.5-day-old buds (stage 5). It changed into a tube sac whose anterior half had cilia on the luminal surface (stage 9, eight days old); the ciliated duct thus formed. In 6-day buds (stage 8), a large cell mass could be observed histologically beneath the neural complex. The cell bodies were soon arranged at the periphery of the cell mass, thus forming the dorsal ganglion. Rudiments of the pericardium, gonad, and pyloric duct appeared first as small aggregates of mesenchymal cells at stages 7, 8, and 11 (12 days old), respectively. Muscle precursors appeared in the mesenchymal space in association with the epidermis at stage 11. They differentiated into the musculus sphincter and longitudinal body musculature during stage 12, at which time, too, the stigmata perforated the pharyngeal wall. Zooids began to feed and thus attained functional maturity after about two weeks of development from buds.

INTRODUCTION

In botryllid and polystyelid ascidians palleal buds are formed by evagination of both the epidermis and peribranchial epithelium of the parental zooid (Berrill, 1940, 1941, 1947, 1948; Abbott, 1953; Fujimoto and Watanabe, 1976; Kawamura and Watanabe, 1982a). Since *situs inversus* zooids were experimentally induced in *Botryllus schlosseri* (Sabbadin, 1956), palleal buds have been used as excellent materials to investigate the development of body axes and body pattern (Izzard, 1973; Sabbadin *et al.*, 1975; Kawamura and Watanabe, 1982b, c; Nakauchi and Sugino, 1984). Recent works (Kawamura and Watanabe, 1983; Kawamura, 1984) have shown that in *Polyandrocarpa misakiensis* the anteroposterior (A-P) axis of a bud is determined with the help of positional information in the parental mantle wall. In contrast with increasing experimental works, descriptive studies on the development of zooid organization have not advanced beyond Izzard's work (1973), although fragmentary knowledge is accumulating (*e.g.*, Nunzi *et al.*, 1979; Kawamura and Nakauchi, 1984).

Symplegma reptans is one of the common botryllid ascidians in Japanese waters.

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* This paper is dedicated to the memory of the late Professor Donald P. Abbott, with deep respect.

TABLE I

Developmental stages of palleal buds in Symplegma reptans *based on living and fixed specimens*

Stage	Living materials	Fixed materials
0	Bud primordium	
1	Maximal size and evagination of bud primordium	
2	Formation of hemisphere	Proximo-distal gradient of cell height
3	Formation of closed vesicle, appearance of long axis	
4	Evagination of epidermis (tv_2), rupture of epidermal stalk (tv_1), appearance of body axes (concavities and convexities in inner vesicle)	Bilateral asymmetry of cell thickening
5	Neural complex placode forms	Presumptive domain of gut rudiment
6	Development of atrial folds and posterior projections	Establishment of budding zone
7	Formation of gut rudiment	Appearance of pericardium and gonadal cell mass
8	Pericardium, gonadal rudiment, and bud primordium appear	Dorsal ganglion cells, endostylar placode
9	Evagination of first bud, appearance of siphon anlagen	Ciliated duct, completion of pharyngeal wall, elaboration of gut rudiment
10	Appearance of endostyle	Anlagen of stigmata
11	Heartbeat begins, tv_3 and tv_4 form	Cell mass of pyloric duct
11′	Normal heartbeat	
12	Appearance of stigmata	Formation of body musculature
13	Stigmatal ciliary movement, functional maturity	
14	Sexual maturity	
15	Degeneration	

Its availability for the study of developmental biology has been pointed out repeatedly by the junior author of this paper (*cf.*, Sugimoto and Nakauchi, 1974; Nakauchi, 1976). This species is very transparent, easy to handle, and amenable to experimental manipulation (Sugimoto and Watanabe, 1980). Unfortunately, a full account of bud development in *Symplegma* has never been reported, although several workers have suggested that the basic strategy of body patterning is similar to that of *B. schlosseri* (Berrill, 1940; Sugimoto and Nakauchi, 1974; Kawamura and Nakauchi, 1976; Kawamura and Watanabe, 1982b).

The purpose of this study is to give a detailed account of the development of palleal buds in *S. reptans.* This has been accomplished by the daily observation of living animals and histological study of fixed animals. Here we present the developmental stages and timetable and report new findings in ascidian budding. We also present comparative analysis of budding in *S. reptans* as compared with blastogenesis in other botryllids and polystyelids.

MATERIALS AND METHODS

Colonies of *Symplegma reptans,* which had been cultured in the bay in the vicinity of the Usa Marine Biological Institute of Kochi University, were used as the source of larvae. Taxonomic descriptions, life history, and culture methods will be found in Tokioka (1949), Sugimoto and Nakauchi (1974), and Nakauchi (1976), respectively.

Both living and fixed specimens were studied. Observation was made on young colonies (about one week after metamorphosis) in June (seawater temperature, 20–22°C), in November (22–24°C), and in December (18–20°C). In order to observe the

TABLE II

Timetable of bud development in Symplegma reptans *at different temperature conditions*

	Time schedule		
	Inland culture		Field
	18°C	22°C	18°–22°C
Stages	(hours)	(hours)	(days)
1	0	0	0
2	24.8	13.0	1.1
3	53.5	24.9	2.1
4	70.9	36.0	2.9
5	78.0	46.9	3.4
6	97.0	54.7	4.0
7	112.3	61.3	5.1
8	133.6	73.0	6.2
9	156.0	81.3	7.7
10	202.5	96.0	9.3
11	231.9	116.7	12.1
12	266.8	138.0	13.4
13	311.1	162.0	15.8

development of individual buds successively, colonies attached to a glass plate were cultured in a small tank placed in the laboratory. The seawater in the tank was changed twice each day. Observations were made on individual buds at intervals of eight hours. All the drawings were made with the aid of camera lucida. The rate of bud development of colonies in the laboratory was compared with that of colonies in the bay once a day. In order to facilitate histological preparations some colonies were cultured on polyethylene film. The materials were anethetized with menthol for 2 hours and fixed with Bouin's solution dissolved in seawater. They were whole-stained with borax carmine, then sectioned at 5 μm and stained with Delafield's hematoxylin and eosin.

RESULTS

In our study the life span of blastozooids was divided into 15 developmental stages (Table I), of which stage 14 and stage 15 are involved in sexual maturation and zooid degeneration, respectively. As those later developmental events are beyond the purpose of this study, only bud development up to stage 13 is dealt with here. The timetables of bud development at different temperature conditions are given in Table II. The major developmental events were observed to occur in sequence as follows.

Stage 0 and stage 1

The earliest morphological sign of bud formation was observed in the right anterior region of the atrial epithelium of a developing parental zooid when that zooid was at stage 8 (Figs. 1A, 2E:10–16). Cells of the presumptive bud domain gradually became thickened to a maximal thickness of about 10 μm (*cf.*, Fig. 3). The bud primordium in 5 μm sections consisted of about 20 cells. In this species 3–6 buds are formed in sequence from this thickened area (Sugimoto and Nakauchi, 1974), and so it is practically impossible to determine the very start of bud formation except for the first bud of each series. In the present paper, therfore, stage 1 of bud development was allocated to a short period when the maximal thickness of the primordium was attained. The still-thickening bud primordium before this stage was defined as stage 0. The duration of stage 0 differs among buds, depending on their order of appearance in the series.

Stage 2

In this stage a bud hemisphere is formed by arching of the bud primordium (Figs. 2II, 4A). The protruding peribranchial wall is thickest at the distal area, and thinnest in the proximal part. The hemisphere consists of about 30 cells per 5 μm section. The luminal surface of the apical wall becomes indented (Fig. 4A). Nuclei are located on the outside of the thickened epithelium.

Stage 3

Stage 3 is characterized by the contraction of the basal area of the peribranchial hemisphere, a closed inner vesicle thus being formed (Figs. 1B, 2III). The outer and inner epithelia of the bud are derived from the epidermis and atrial epithelium of the parental zooid, respectively. The inner epithelium consists of about 50 cells per 5 μm section. Now the closed vesicle is ellipsoid in outline (Fig. 2III) with the long axis 1.2 times (outer layer) and 1.3 times (inner layer) the short axis. The long axis of the first bud is nearly parallel to the anteroposterior axis of the parental zooid in most cases, but often skewed in later buds. The inner layer is always thickened on the side corresponding to the right side of the future zooid (Fig. 2III). This difference in thickness of the inner wall persists through several subsequent stages (*cf.*, Fig. 1C).

The proximal end of the inner wall bears the atrial stalk on the left side of the long axis (Fig. 2III). The outer epidermal layer was not closed completely, but connected to the parent basally (Figs. 1B, 2III). This stalk acted as a test vessel, here referred to as test vessel 1 (tv_1). Unlike *Botryllus,* the tv_1 is not involved in the transfer of blood cells.

Stage 4

The second test vessel (tv_2) is formed during stage 4 from a diverticulum of the bud epidermis (Figs. 1C, 2IV), the tip of which fused with the common vascular system in the tunic. It was through this tv_2 that a developing bud was supplied with active blood circulation. Although Izzard (1973) observed a well-defined channel of bloodflow in a young bud of *Botryllus schlosseri,* no such channel was found in the present species. In contrast with the development of tv_2, tv_1 soon began to atrophy, and finally it was absorbed completely by the bud (Fig. $2IV^+$).

Body axes become gradually evident at this stage. The tv_2 developed into a ventral vessel of the future zooid, thus defining the dorsoventral (D-V) axis. As the vessel usually grew out toward the substratum, the D-V axis coincided with the parental axis. A few small morphological changes took place on the inner vesicle. Two concavities, the rudiment of pharyngeal fold, were formed sequentially on the right and left sides of the long axis (Figs. 1C, 2IV, IV^+). The vestigial atrial stalk remained as a small projection on the inner vesicle (Figs. 1C, 2A, IV^+) with an additional projection soon appearing on its right side. These projections developed into the right and left posterior ends of the peribranchial wall (PEP), respectively (see below). Thus, these projections and concavities define the anteroposterior axis of the developing bud which coincides roughly with the long axis. The posterior end of this axis was shifted

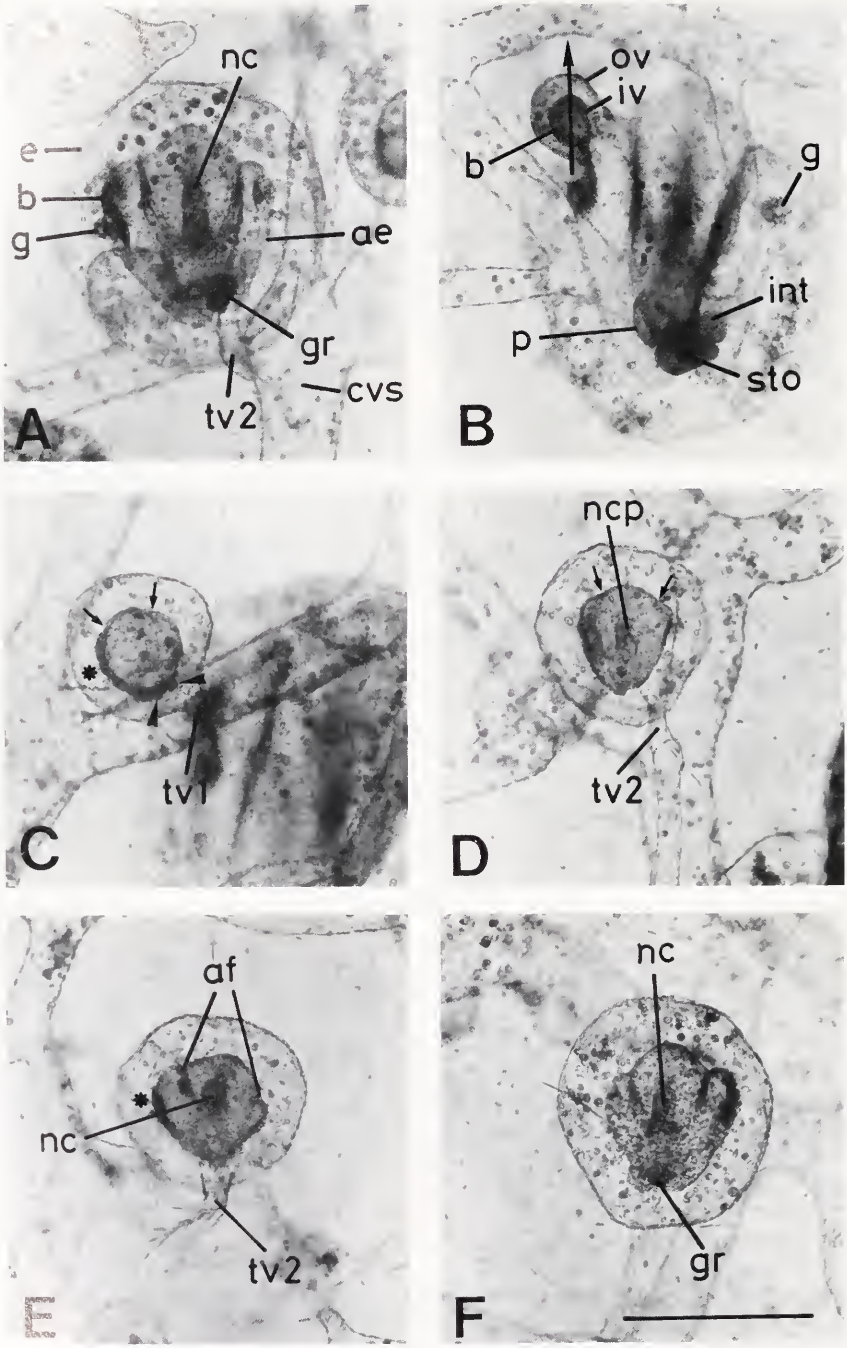
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FIGURE 1. Photographs of bud development of *Symplegma reptans*, fixed whole-mounts. (A) The stage 0 bud primordium on a parental bud of stage 8, ventral view. (B) Early stage 3 bud vesicle on a parental bud of stage 10, ventral view. Arrow indicates long axis. (C) Stage 4, ventral view. Arrows in inner vesicle indicate anlagen of atrial folds. Arrowheads indicate anlagen of right and left posterior ends of peribranchial wall. Note that one side of the inner wall (asterisk) is thicker than the other side. (D) Stage 5,

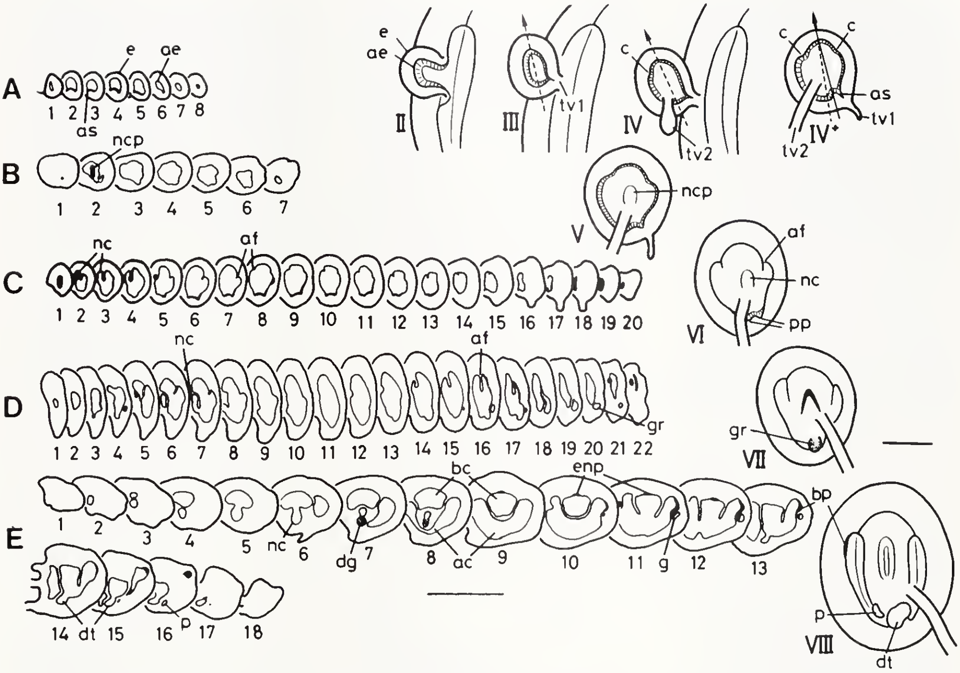
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FIGURE 2. Serial sections of buds from stage 4 to stage 8 (left), and ventral views of living buds from stage 2 to stage 8 (right). Roman numerals show developmental stages. Arrow with solid shaft, anteroposterior axis; arrows with broken shaft, long axis. (A) Stage 4, from dorsal to ventral. (B) Stage 5, from dorsal to ventral. (C) Stage 6, from right dorsal to left ventral. (D) Stage 7, from right to left. (E) Stage 8, from dorsal to ventral. ac, atrial chamber; ae, atrial epithelium; af, atrial fold; as, atrial stalk; bc, branchial chamber; bp, bud primordium; c, concavity; dg, dorsal ganglion; dt, digestive tract; e, epidermis; enp, endostylar placode; g, gonad; gr, gut rudiment; nc, neural complex; ncp, neural complex placode; p, pericardium; pp, posterior projection; $tv_{1,2}$, test vessel 1, 2. Bars = 0.2 mm.

somewhat to the left of the long axis. The mid-ventral line of *Symplegma* zooids curves leftward (toward the stomach) near the posterior end, thus showing bilateral asymmetry (*e.g.*, Fig. 3A). The origin of this asymmetry could be traced back to stage 4.

During this stage, the bud expands bilaterally and the long axis becomes unclear.

Stage 5

Stage 5 begins with the appearance of the neural complex placode (Figs. 1D, 2B). This primordium, formed as a thickened disc of cells at the center of the dorsal wall

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ventral view. Placode of neural complex appears on dorsal side. Concavities (arrows) have not yet developed. Bud is connected with the common vascular system only through tv_2. (E) Stage 6, ventral view. Atrial folds begin to invaginate. Note that right anterior region of atrial epithelium (asterisk), presumptive domain of budding zone, has already thickened. (F) Stage 7, dorsal view. Gut rudiment appears. ae, atrial epithelium; af, atrial folds; b, bud; cvs, common vascular system; e, epidermis; g, gonad; gr, gut rudiment; int, intestine; iv, inner vesicle of bud; nc, neural complex; ncp, neural complex placode; ov, outer vesicle of bud; p, pericardium; sto, stomach; $tv_{1,2}$, test vessel 1, 2. Bar = 0.5 mm.

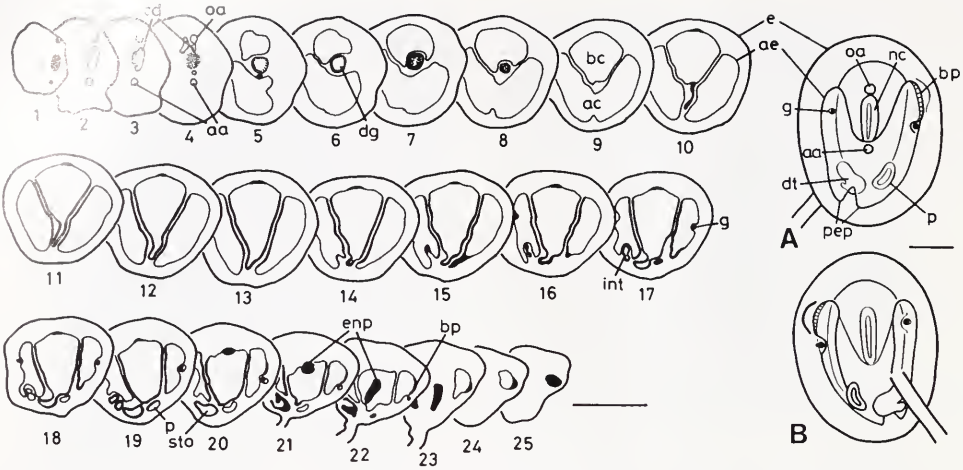

FIGURE 3. Developing buds of stage 9. (Left) Serial sections from dorsal to ventral. (Right) Living animals viewed from dorsal side (A) and ventral side (B). aa, atrial aperture; ac, atrial chamber; ae, atrial epithelium; bc, branchial chamber; bp, bud primordium; cd, ciliated duct; dg, dorsal ganglion; dt, digestive tract; e, epidermis; enp, endostylar placode; g, gonad; int, intestine; nc, neural complex; oa, oral aperture; p, pericardium; pep, posterior end of peribranchial wall; sto, stomach. Bars = 0.2 mm.

of the inner vesicle, is the first organ rudiment to appear. Cells situated between the right and left PEP anlagen became also thickened. They were the presumptive domain of the gut rudiment.

Stage 6

At the beginning of stage 6, the pharyngeal (atrial) folds originating from the concavities of the inner vesicle grow posteriorly (Fig. 1E), and the PEP anlagen become more discernible during this stage (Fig. 2C, VI). In living specimens, cells of the inner epithelial wall appeared squamous on each side of the body (Fig. 2VI). However, fixed whole mounts disclosed thickening of the right anterior region of the presumptive atrial epithelium at this and subsequent stages (Fig. 1E, F). The neural complex took on a rod-like shape (Fig. 2C:1–5). Both its ends were open to the cavity of the inner vesicle.

Stage 7

Stage 7 is recognized by the appearance of the gut rudiment (Figs. 1F, 2VII) between the two projections developing into the right and left ends of the posterior peribranchial wall. The pharyngeal folds continued to grow toward the posterior projections (Figs. 2D:5–9, 2D:14–18, 4B). A small vesicle, the eventual pericardium, appears in sections near the gut rudiment (Fig. 4B). In no case did we see the pericardial rudiment to be formed as an evagination of the inner vesicle. The dorsal ganglion was not yet established.

Stage 8

At stage 8 the pericardial rudiment can be first recognized in living specimens (Fig. 2VIII). Beginning as a single-walled vesicle, it was changed into a double-walled

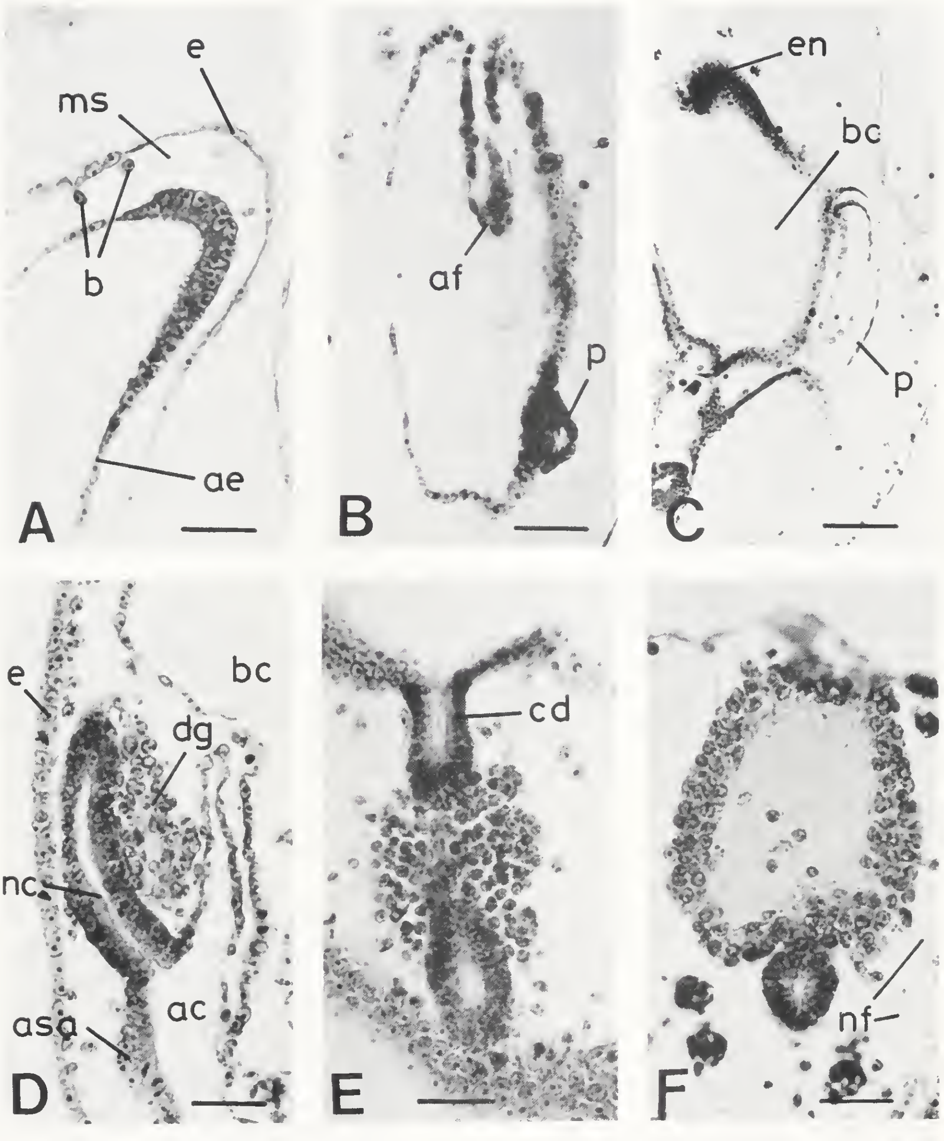

FIGURE 4. Photographs of histogenesis. (A) Protruding bud of late stage 1, frontal sectioning. Bar = 20 μm. (B) Inner wall of a developing bud of stage 8, longitudinal sectioning. Bar = 20 μm. (C) Double-walled pericardium of stage 9, frontal sectioning. Bar = 40 μm. (D) Neural complex and dorsal ganglion cells of late stage 8, longitudinal sectioning. Bar = 20 μm. (E) Neural complex of stage 9, frontal sectioning. Its anterior half is opened to the pharyngeal chamber and has cilia on the luminal surface. Bar = 20 μm. (F) Dorsal ganglion of stage 11, frontal sectioning. Bar = 20 μm. ac, atrial chamber; ae, atrial epithelium; af, atrial fold; asa, atrial siphon anlage; b, blood cell; bc, branchial chamber; cd, ciliated duct; dg, dorsal ganglion; e, epidermis; en, endostylar placode; ms, mesenchymal space; nc, neural complex; nf, nerve fiber; p, pericardium.

vesicle during this stage (*cf.*, Fig. 4C) by inward folding of the cardiac muscle cell domain. The pericardial rudiment was situated to the right of the gut rudiment, making clearer the bilateral asymmetry of the earlier stages.

The atrial (pharyngeal) folds extend to the posterior end of the inner vesicle on the ventral surface (Fig. 2E:13–15), while meeting just behind the neural complex on the dorsal surface (Fig. 2E:8), thus separating the branchial chamber from the atrial chamber. The anterior and posterior ends of the neural complex were opened into the branchial and atrial chamber, respectively (Figs. 2E:5–6, 4D). Free cells began to accumulate beneath the neural complex to form the dorsal ganglion cells. In sections the endostyle rudiment appears as a cell thickening along the mid-ventral line of the branchial floor (Fig. 2E:9–12).

By mid stage 8 of living animals a stage 0 bud primordium becomes visible on the right anterior region of their atrial epithelium (Fig. 2VIII). However, as we have already described, thickening of the right atrial epithelium could be traced back at least to stage 6 in sections or fixed whole mounts.

The gonadal rudiment, derived from a small mass of cells observed in sections of the preceding stage, became visible in living animals on each side of the body. It was located within a concavity, termed the genital cavity, formed by an invagination of the peribranchial wall (Figs. 2E:10–13, 5A, B). The dextral genital cavity was situated just posterior to the bud primordium (Fig. 5B). The sinistral one was situated a little more anteriorly than the dextral. The gonadal rudiment itself took a hollow, spherical form. A multi-layered mass of cells faced the wall of the genital cavity. They contained oocytes with the largest nuclei about 5 μm in diameter (Fig. 5A). The testis also develops from this cell mass on the dorsal side of the oocytes (not shown). On the other hand, gonadal cells facing the mesenchymal space were mono-layered (Fig. 5B). They form the brood pouch and oviduct at later stages. The gonad did not undergo substantial further morphological changes until the colony entered the sexual reproductive phase. Further development of the gonad will be described in another report.

Stage 9

At stage 9 the bud primordium begins to evaginate. Two siphon anlagen which were first seen in sections of stage 8 specimens could now be observed on the mid-dorsal line of the "parental" bud near each end of the neural complex (Fig. 3A). The branchial siphon was formed by the contact of wall of the branchial (pharyngeal) chamber with the epidermis, while the atrial siphon by the contact of wall of the atrial chamber with the epidermis (*cf.*, Fig. 4D).

The branchial chamber was now completely separated from the atrial chamber (Fig. 3:9–22). Around this stage, the neural complex lost its connection with the atrial chamber. The anterior half of the neural complex had cilia on its luminal surface, which was differentiating into the ciliated duct (Fig. 4E). Cytoplasm facing the lumen of the ciliated duct was weakly stained with eosin. In the dorsal ganglion, cell bodies became peripherally arranged, and the central matrix of the ganglion was eosinophilic (Fig. 3:5–6). The gut rudiment elongated and began segmentation into the esophagus, stomach, pyloric caecum, and intestine.

Stage 10

At the beginning of stage 10 the endostylar rudiment becomes visible in living specimens. This had already been recognized histologically at stage 8 but was not evident in living specimens until the longitudinal groove of the endostyle emerged mid-ventrally on the pharynx (*e.g.*, Fig. 6:45–48). Usually the dextral margin of the groove appeared earlier than the sinistral one (Fig. 6A, B).

The pharynx consists of two epithelial walls; one facing the branchial chamber and the other the atrial chamber. In sections, cells of the walls began to be swollen at intervals along the anteroposterior axis (*e.g.*, Fig. 6:29–32). These were the first sign of the stigmata anlagen. In the alimentary tract the stomach was especially enlarged, but not yet plicated.

The first bud of the "parental" bud had by now developed into stage 2.

Stage 11

Stage 11 begins with the start of the heartbeat. At first the heartbeat was faint and irregular. The stage where the heart beats regularly is defined as stage 11′. A parental zooid of this stage bore its own first bud of stage 4 and the second one of stage 1 (Fig. 7A).

Until stage 11 a developing zooid had only one test vessel, tv_2, but now tv_3 appeared as an evagination of the ventral epidermal wall near the posterior end and tv_4 appeared beneath the budding zone (Fig. 7). Both vessels elongated and connected with the common tunic vascular system.

The ventral edge of the branchial sac looked wavy (Fig. 7A). In sections, the outer and inner pharyngeal walls were not only thickened at intervals but also associated tightly with each other (*e.g.*, Fig. 7:21–24). These stigmata anlagen could not be detected in living specimens until stage 12. In the digestive tract, cell aggregates appeared in the vicinity of the descending limb of the intestine (Fig. 5C). During this and subsequent stages, they developed into the pyloric gland and duct (Fig. 5D). The wall of the stomach now began to show plications.

The dorsal ganglion extended nerve fibers (Fig. 4F). The luminal surface of the ciliated duct became highly eosinophilic. Single basophilic mesenchyme cells became associated with the dorsal epidermis and both siphons (Fig. 5E). They were small and spherical around the siphons, but often fusiform beneath the epidermis. These cells were the precursor of body musculature.

Stage 12

Stage 12 is defined by perforation of the stigmata. The stigmata grew from the wave-like structures of the branchial wall (Fig. 7B), each "wave" coinciding with a stigmatal row where the pharyngeal wall had been thickened at stages 10 and 11. The antero-ventral four to six rows of stigmata appeared first, and new ones were added postero-dorsally up to 7–8. The internal longitudinal vessels could first be seen at this stage. At first, only one stigma was perforated between respective vessels (Fig. 7B), and then two or three stigmata were added to form a total of 16–19 stigmata per row. It was unclear whether the perforation of stigmata was accompanied by cell dissociation or cell death of the thickened cells of the pharynx.

Muscular sphincters appeared around the oral and atrial siphons, and the longitudinal body muscles were developed just beneath the dorsal epidermis (Fig. 5F). Muscular cytoplasm was highly eosinophilic. Glandular cells containing eosinophilic granules appeared in the posterior part of the neural complex, forming the neural gland.

Stage 13

At stage 13 the cilia of the stigmata began to beat, and feeding commenced one or two days later. Thus the young zooids were functional.

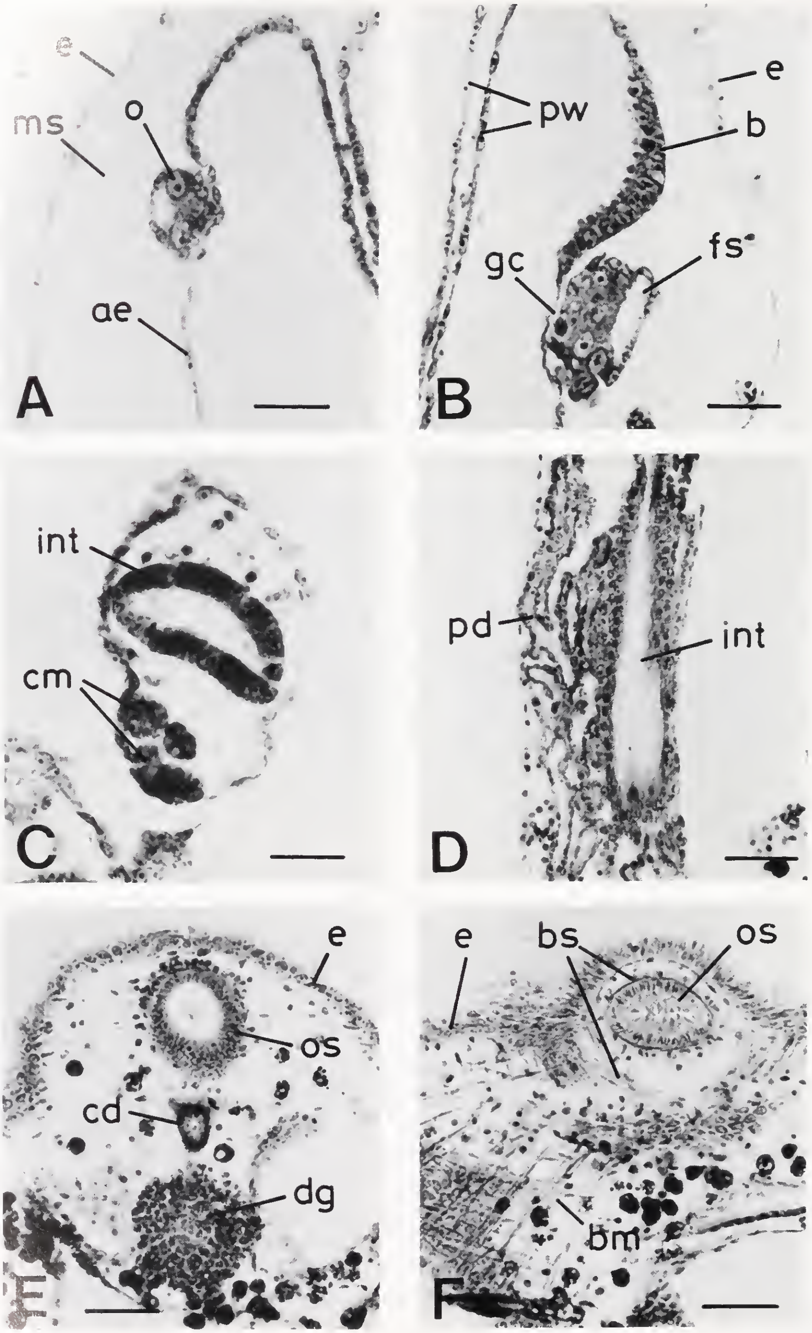

FIGURE 5. Photographs of histogenesis. (A) Sinistral gonadal rudiment of stage 8, frontal sectioning. Bar = 20 μm. (B) Dextral gonadal rudiment of early stage 9, frontal sectioning. Bar = 20 μm. (C) Descending limb of intestine, stage 11, transverse sectioning. A large number of small cell mass appear. Bar = 20 μm. (D) Intestine and pyloric duct of stage 12, frontal sectioning. Bar = 40 μm. (E) Dorsal surface of a developing zooid of stage 11, frontal sectioning. Single mesenchymal cells are associated with the epidermis

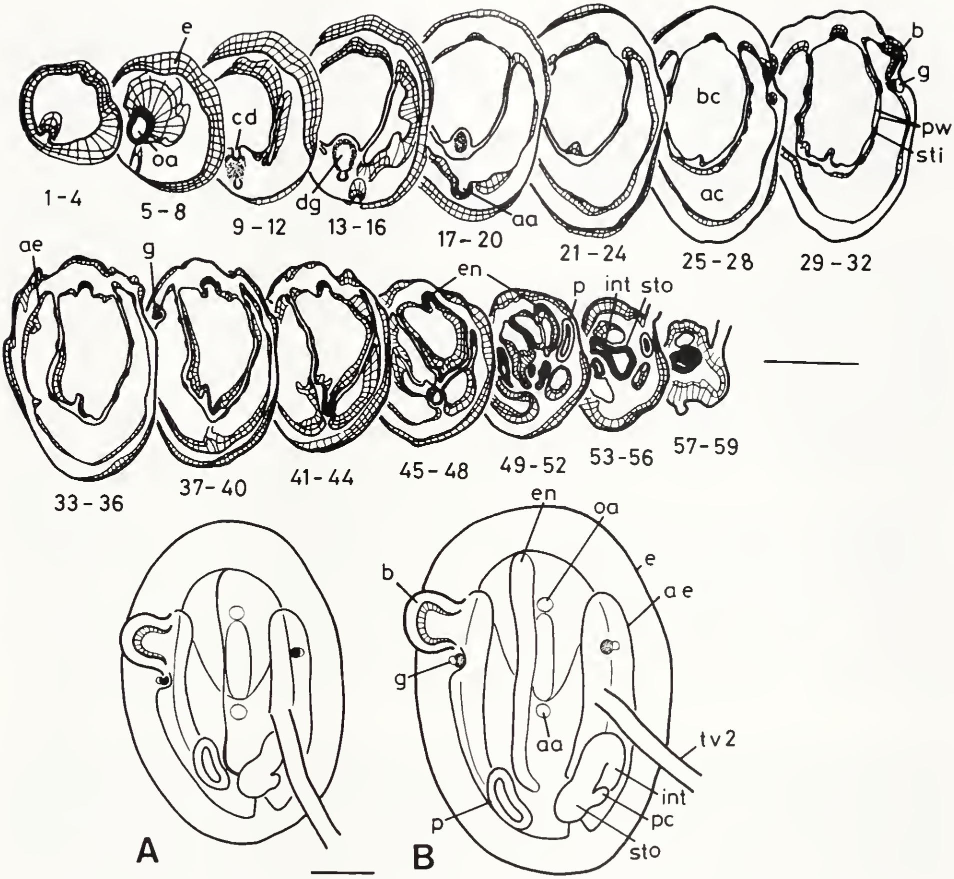

FIGURE 6. Developing zooids of stage 10. (Upper) Serial sections from dorsal to ventral. (Lower) Living animals of early stage 10 (A) and late stage 10 (B), ventral view. aa, atrial aperture; ac, atrial chamber; ae, atrial epithelium; b, bud; bc, branchial chamber; cd, ciliated duct; dg, dorsal ganglion; e, epidermis; en, endostyle; g, gonad; int, intestine; oa, oral aperture; p, pericardium; pc, pyloric caecum; pw, pharyngeal wall; sti, stigmata anlagen; sto, stomach; tv_2, test vessel 2. Bars = 0.2 mm.

DISCUSSION

In the present study, we have revealed the developmental stages of a bud and their time schedule in *Symplegma reptans*. The results give new information about the difference between *Symplegma* and other botryllid and polystyelid budding.

Formation of the bud primordium

In *Botryllus schlosseri* the bud primordium first appears as a thickened disc of cells on each side of the parental body of stage 8 (staging by Izzard, 1973). In contrast,

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and the oral siphon. Bar = 40 μm. (F) Dorsal surface of a developing zooid of late stage 12, frontal sectioning. The branchial sphincter and body muscle are developed. Bar = 40 μm. ae, atrial epithelium; b, bud primordium; bm, body muscle; bs, branchial sphincter; cd, ciliated duct; cm, cell mass; dg, dorsal ganglion; e, epidermis; fs, follicle stalk; gc, genital cavity; int, intestine; ms, mesenchymal space; o, oocyte; os, oral siphon; pd, pyloric duct; pw, pharyngeal wall.

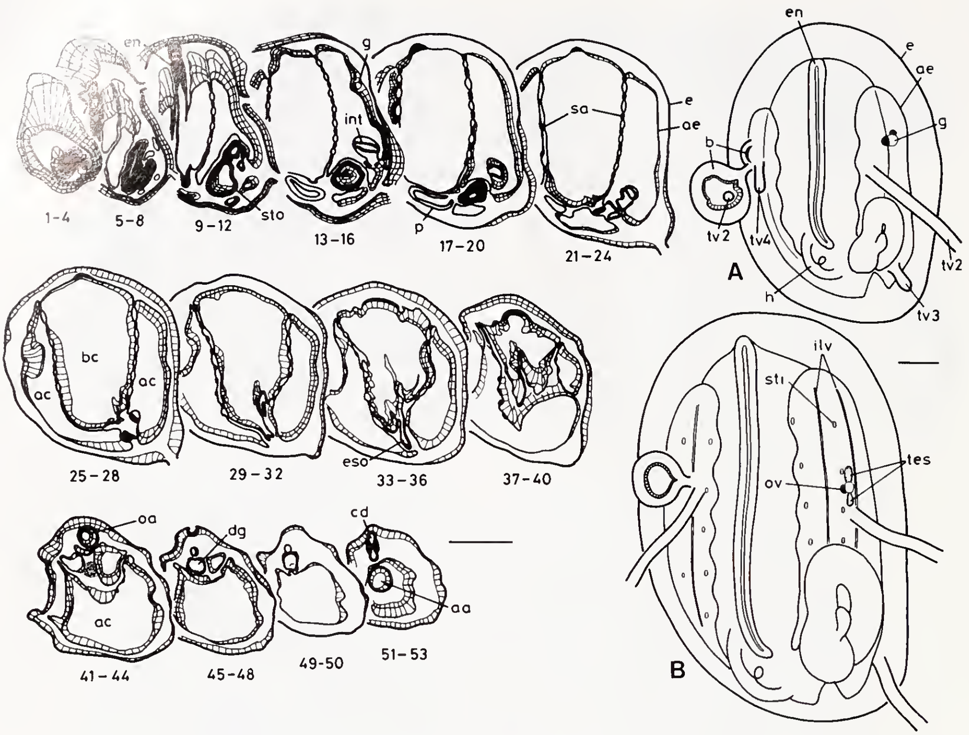

FIGURE 7. Developing zooids of stages 11 and 12. (Left) Serial sections of stage 11 zooid, from ventral to dorsal. (Right) Living animals of stage 11 (A) and stage 12 (B), ventral view. aa, atrial aperture; ac, atrial chamber; ae, atrial epithelium; b, bud; bc, branchial chamber; cd, ciliated duct; dg, dorsal ganglion; e, epidermis; en, endostyle; eso, esophagus; g, gonad; h, heart; ilv, inner longitudinal vessel; int, intestine; oa, oral aperture; ov, ovary; p, pericardium; sa, stigmata anlagen; sti, stigmata; sto, stomach; tes, testis; $tv_{2,3,4}$, test vessel 2, 3, 4. Bars = 0.2 mm.

S. reptans shows cell thickening, the first manifestation of budding, at a much earlier stage. Developing buds of stage 6 had already showed significant cell thickening on the right anterior region of the atrial epithelium. The area of thickening eventually could be traced back to the distal thickened area of a protruding bud (stage 2). Our unpublished data suggest that in *S. reptans* columnar or cuboidal cells are physiologically more active than squamous cells. These results suggest in turn that the budding activity of a developing zooid may not be acquired *de novo* but result from the transmission and maintenance of high physiological activity in the budding zone of the preceding generation. *S. japonica* in which a bud arises on each side of the parental body (Watanabe, pers. comm.) may contribute to our further understanding of the manner by which the budding domain is established.

Unlike *Botryllus* and *Botrylloides* zooids, each *Symplegma* zooid makes buds several times during its life span (Sugimoto and Nakauchi, 1974). The area of the thickened disc of *Symplegma* is not wholly involved in a single bud, but some area remains as sources of the succeeding buds (Berrill, 1940). The duration of the disc stage is, therefore, short in earlier buds but has become longer and longer in later buds. This makes it practically impossible to apply the staging of *Botryllus* (Sabbadin, 1955; Izzard, 1973) to *Symplegma* without major modification. In order to compare the developmental timetables of successive buds, we distinguished a growing bud disc from the fully developed disc, by defining the former as stage 0. Our stage 0 and stage 1, therefore, correspond to stage 1 and stage 2 of Berrill (1941). Stage 1 as defined by Izzard (1973) covers our stages 0 and 1. Stage 0 is histologically detectable when the parental bud enters stage 6.

Development of bud polarities and bilateral asymmetry

Symplegma differs from *Botryllus* in the way the closed vesicle is formed from a hemisphere. In *B. schlosseri,* the beginning of stage 3 is recognized by the skewing of the atrial hemisphere in the sagittal plane toward the anterior end of the parental bud (Izzard, 1973). The long axis of the resultant bud vesicle is in harmony with the A-P axis of the parent, and the dorso-ventral (D-V) axis lies perpendicular to the parent in a horizontal plane (Sabbadin *et al.,* 1975). Vesicle formation in *S. reptans* varies from individual to individual. The long axis of a stage 3 bud is not always parallel with the parental A-P axis. The D-V axis that orients differently according to case, works in harmony with that of the parent by the time the bud's neural complex is formed (Kawamura and Watanabe, 1982b). However, we do not think that this difference comes from an intrinsic difference in bud formation but from the fact that *Botryllus* forms a common cloacal system and each bud is settled in a fixed position in the system at the early stage of its development. *Symplegma,* on the other hand, makes no systems and buds are able to change their position and orientation in the colony as late as stage 12. Because of this capacity for movement, a *Symplegma* bud can easily change the orientation of its long axis and D-V axis relative to those of the parent.

Previous work (Kawamura and Watanabe, 1982b) has shown that in *S. reptans* bud polarity develops as if the bud "knew" the position on the parental lateral wall from which it has arisen, a phenomenon referred to as the parental lateral effect. The present results confirm and extend our earlier findings histologically. The thickened columnar cells of the apical region of a stage 2 bud could be followed until stage 4, when the antero-posterior polarity became visible. They constituted mainly the dextral lateral wall of a developing bud. The atrial stalk that had connected the inner vesicle with the parental peribranchial wall formed a small projection and finally developed into the posterior end of the left peribranchial wall (left PEP) in the future zooid. In *B. schlosseri,* two posterior corners of inner vesicle appear in advance of the atrial folds (the pharyngeal rudiment), and the right posterior corner projects further posteriorly than the left one (Izzard, 1973). This is the first morphological expression of bilateral asymmetry. Our PEP anlagen are different from the posterior corners of *Botryllus* in their sites of appearance and in their mode of development. In *Botryllus* the posterior corners form on each side of the atrial stalk, and later the right one grows further than the left one. This unequal growth results in a skewing of the body axis toward the left side posteriorly in the future zooid. In *Symplegma,* as the left PEP anlage corresponds to the atrial stalk, the right side of the body is larger than the left side from the very start. Thus, we conclude that the skewing of body axis in *Symplegma* is associated with the asymmetrical emergence of PEPs.

In *B. schlosseri,* experimental work has suggested that bud polarity is determined by the common vascular system (Sabbadin *et al.,* 1975). In normally developing buds, the epidermal stalk serves as a blood vessel, and a channel of bloodflow is established within a bud of late stage 3 (Burighel and Brunetti, 1971; Izzard, 1973). The point where the stalk enters the bud corresponds to the posterior end of the future zooid. In *S. reptans* no active circulation could be observed between buds and the parent through the stalk, tv_1, which atrophied at stage 4 (*cf.*, Mukai *et al.*, 1978). Instead, tv_2, which emerged ventrally at stage 4, was the first and only vessel that connected the bud with the common vascular system, until tv_3 and tv_4 appeared at stage 11. The entrance point of blood through tv_2 was the left ventral region of the future zooid. Thus, the contribution of bloodflow to body axis determination seems to be negligible in *S. reptans.*

In *S. reptans,* the posterior end of a developing bud is established in the vicinity of the atrial stalk where cells originally occupying the periphery of the bud primordium come in contact with one another at the time the protruding bud is pinched off. This means that normally nonadjacent cells are juxtaposed at the proximal end of a bud vesicle. In palleal buds of *Polyandrocarpa misakiensis,* such a juxtaposition of cells has been shown to play an important role in the establishment of anteroposterior body pattern (Kawamura and Watanabe, 1983; Kawamura, 1984). When bud pieces of *Polyandrocarpa* originating from different positions of the parental mantle wall are surgically apposed, mitotic activity is enhanced at the boundary region, resulting in the formation of the gut rudiment that is the posterior-most organ (Kawamura and Nakauchi, 1986). We are currently trying to determine whether the posterior determination in *Symplegma* buds is of *Polyandrocarpa* type.

Basic strategy of organogenesis

The pharyngeal rudiment, gut rudiment, endostyle, and neural complex are the major organ rudiments formed directly from the inner vesicle of a bud in *S. reptans.* According to Berrill (1941) and Izzard (1973), the gut rudiment is the first organ to be established in a developing *Botryllus* bud, and then the rudiments of the neural complex and pericardium follow. In *Metandrocarpa taylori,* the endostyle is one of the first structures to appear in the newly detached bud (Abbott, 1953). In *S. reptans,* the neural complex placode manifested itself at stage 5, and then the gut rudiment appeared (stage 7), well before the endostyle (stage 10). The atrial folds (pharyngeal rudiment) had not yet developed at stage 5. This precocious appearance of the neural rudiment in *Symplegma* is instructive when considered from the comparative embryological viewpoint. In colonial ascidians, zooid organization can be constructed via two ways; one from an embryo or tadpole larva and the other from an asexual bud. Presently we do not know the extent to which these two pathways share the basic strategy of organogenesis with each other. But, in this study we can find an analogy between the neural complex tube in *Symplegma* buds and the "neural tube" in ascidian embryos. In chordate embryogenesis, the neurula stage follows the gastrula. As the double-walled vesicle stage (stage 3) of ascidian palleal buds is comparable to the gastrula stage of embryos (*cf.*, Berrill, 1955), the stage 5 of *Symplegma* may be likened to a "neurula" stage. Recentry, we found that in *Botrylloides simodensis* blastogenesis (in prep.) the neural placode was the earliest organ rudiment to be established, paralleling our finding in *S. reptans.*

In *Botryllus* the neural complex arises as a tubular dorsal outpocketing of the inner vesicle (Hjort, 1896; Ritter, 1896; cited by Abbott, 1953). This outpocketing grows forward and establishes a secondary connection anteriorly with the pharyngeal cavity, the original posterior aperture soon closing permanently. In *M. taylori,* the tube arises as a folded placode which cuts off starting from the anterior end, and the definitive anterior connection with the pharyngeal cavity is primitive, not secondary (Abbott, 1953). In *S. reptans,* the ridge of the neural complex placode begins to cut off as a separate tube, except at each end, where a small connection with the inner vesicle remains. Thus, the process of neural rudiment formation in *Symplegma* is similar to that in *Metandrocarpa*. In *M. taylori*, the extreme posterior tip of the neural complex is cut off at a fairly early stage, before any trace of the brain appears (Abbott, 1953). In *S. reptans*, on the other hand, the loss of the connection occurred at stage 9, much later than the brain formation.

The dorsal ganglion of *M. taylori* arises from the zone of junction of the neural complex and the inner vesicle and also from the ventral and ventrolateral walls of the neural complex already separated from the inner vesicle anteriorly and posteriorly (Abbott, 1953). In *S. reptans* the dorsal ganglion formed only after the neural complex had been separated completely from the inner vesicle, and therefore probably not in a zone of junction like *Metandrocarpa*'s.

The pericardium, gonad, pyloric duct, and muscle cells of *S. reptans* had their cellular origin elsewhere than in the inner vesicle of the bud. The pericardium and pyloric duct call for some discussion. Earlier works described the origin of the pericardial anlage from the floor of the inner vesicle in botryllids (Oka, 1892; Pizon, 1893; cited by Abbott, 1953; Berrill, 1941, 1947) and in *S. viride* (Berrill, 1940). Abbott (1953) stated that there is no doubt that in *M. taylori* the pericardial anlage arises from the inner vesicle, although he did not observe the very beginning of formation of the pericardial rudiment. On the other hand, recent workers do not believe that the pericardium evaginates from the inner vesicle of *Botryllus* (*e.g.*, Izzard, 1973; Nunzi *et al.*, 1979). And in *P. misakiensis* the pericardium arises as mesenchymal cell mass that has no contact with the inner vesicle (Kawamura and Nakauchi, 1984). Our study of *S. reptans* also indicated a mesenchymal origin of the pericardium.

According to Abbott (1953), the pyloric duct arises from the pyloric caecum and grows forward, penetrating to the mesenchyme surrounding the intestinal loop in *M. taylori* and *Botryllus*. Our data for *S. reptans* are inconsistent with his account. In *S. reptans* the pyloric duct and gland around the intestine are clearly derived *in situ* from the mesenchymal cell mass, although we do not deny the existence of the duct in the vicinity of the pyloric caecum. We have observed that in *B. simodensis* (in prep.), cells with high mitotic activity fuse around the intestine to form tubular structures.

In *Botryllus* the gonadal rudiment appears at the closed vesicle stage (Berrill, 1941). In this genus developing oocytes and an undifferentiated cell mass from which the testis will develop migrate from parental buds to their youngest budding offspring (Mukai and Watanabe, 1976). The germ cells are lodged in the gonadal space between the epidermis and the atrial epithelium just posterior to the budding zone. In *S. reptans* the gonadal rudiment appeared in a rather advanced bud of stages 7–8. It was lodged in the genital cavity, a concavity of the atrial epithelium, which is absent in *Botryllus*. The gonadal rudiment took a spherical, hollow shape and formed directly both sperm and ova and their accessory cells. This single origin of male and female elements contrasts with the dual origin in *Botryllus*. The difference in gonad formation between *Botryllus* and *Symplegma* may be closely related to the differing lifespans of individual zooids in these two genera.

Conclusion

In *S. reptans*, thickened cells of the bud primordium (stage 1) occupy exclusively the right side of a double-walled bud vesicle (stage 3–4). The cell thickening is, then, restricted to the right anterior atrial epithelium, budding domain of the next generation, of a stage 6 bud, suggesting that the budding activity may be transmitted through successive asexual generations. A bud vesicle of stage 3 has the long axis that coincides nearly with the anteroposterior axis appearing at stage 4. But, posteriorly the A-P axis is skewed toward the left side of the long axis, showing that the bilateral asymmetry emerges from the very start. The body organization is constructed mainly by inward and outward foldings of the bud's inner vesicle. The neural complex placode is the first organ rudiment to be established on the inner vesicle (stage 5), which contrasts with its relatively late appearance in *Botryllus.* The pharyngeal and gut rudiments follow the neural rudiment. The pericardium, gonad, pyloric duct, and muscle cells appear first as small aggregates of mesenchymal cells. It is unknown whether the cell mass of respective organ rudiments originates from single stem cells or from heterogenous subpopulations. The gonadal rudiment of *Symplegma* zooids, unlike *Botryllus* zooids, produces both male and female elements.

ACKNOWLEDGMENTS

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FATIGUE DAMAGE: REPEATED LOADING ENABLES CRABS TO OPEN LARGER BIVALVES

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ABSTRACT

Observations of behavior and direct measurements of force indicated that the cancrid crab *Cancer productus* could directly crush only the smallest specimens of *Protothaca staminea,* a venerid bivalve. Crabs opened larger *P. staminea* by repeatedly loading the same region of the bivalve's shell with a chela; we hypothesized that this repeated loading caused fatigue of the shell material. To test whether significant fatigue damage would accumulate in the number of cycles a crab was likely to exert, live bivalves and cleaned valves were cyclically loaded in a mechanical testing machine to loads of a constant maximum amplitude of 70–100% of the bivalves' predicted static strength. Failure frequently occurred in fewer than 200 cycles. Recordings from strain gauges attached to the chelae of crabs showed that during an attack on a bivalve a crab would squeeze more than 200 times and that failure of the bivalve could occur during a force pulse which was weaker than previous force pulses. We conclude that repeated loading enables crabs to open larger bivalves than could be crushed outright; by greatly increasing the maximum size of prey vulnerability this expands the size range of molluscan prey available to crabs.

INTRODUCTION

Crabs can be voracious predators on shelled molluscs and, where abundant, can deplete their preferred prey (Walne and Dean, 1972; Mackenzie, 1977). Hungry crabs will persist in attacking prey that require time-consuming handling techniques. For example, crabs will often open large gastropods by progressively peeling back the body whorl, a much slower method than the rapid crushing of the spire that is possible with smaller gastropods (Zipser and Vermeij, 1978).

Small red rock crabs (*Cancer productus*) can open relatively large venerid bivalves if given sufficient time (Boulding, 1984). The crab braces the bivalve against its body with one chela and uses the other chela to repetitively squeeze the ventral shell margin. The crab may release the shell for a period of time and then reapply its chela, repeating this until a chip breaks off. When a large enough hole has been chipped away, the crab forces one chela between the valves and pries them apart. A more efficient method can be used when the bivalve is small enough to fit within the crab's chela; small bivalves can be crushed outright although those with thick shells may require more than one contraction of the crab's chela. The crab behavior of repeatedly loading bivalve shells was first reported by Elner (1978). He suggested that a shore crab (*Carcinus maenas*), faced with mussels (*Mytilus edulis*) it was not strong

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enough to crush outright, opened them by a cumulative process of extending microcracks that were present in the shell substructure.

The failure of a structure under repeated loading with subcritical loads is known as fatigue failure (Sandor, 1972). In structures made of brittle materials, such as concrete beams and bivalve shells, fatigue damage begins when small cracks are initiated or existing cracks are propagated by a load (Boresi *et al.*, 1978). If the structure is repeatedly loaded and unloaded, these cracks will grow until they reach a size where the structure fails; failure may occur during a load that is less than loads previously applied. Research on concrete beams has shown that the number of loading cycles a beam can withstand is a function of its static strength, but that other factors such as stress concentrations, environmental conditions, and load history can also be important (Anonymous, 1974).

A bivalve shell, like any structure, would be expected to fail if loaded a sufficient number of times (*i.e.*, high cycle fatigue, see Sandor, 1972). The interesting biological question is whether failure will occur within the number of cycles a crab is likely to exert during normal predatory behavior. To test our hypothesis that crabs weaken bivalve shells by fatiguing the shell material we must show that: (1) crabs apply force to bivalve shells in a manner that could cause fatigue damage, and that (2) bivalve shells are susceptible to low cycle fatigue. To do this we measured the magnitude and duration of the force pulses generated by a crab attacking bivalves and used mechanical testing machines to cyclically load bivalve shells under controlled conditions.

MATERIALS AND METHODS

Crab predation behavior

Bivalves [*Protothaca staminea* (Conrad)] and crabs [*Cancer productus* (Randall)] were collected in the vicinity of Bamfield Marine Station, Bamfield, British Columbia. The crabs were held in individual aquaria in free-flowing seawater and observed while preying on a range of sizes (shell lengths 5 mm to 65 mm) of the bivalves.

The magnitude and duration of the forces generated when these crabs attacked the bivalves were recorded from strain gauges attached directly to the crabs' chelae (Elner and Campbell, 1981); in effect we used the cuticle of the crab's chela as a force transducer. These strain gauges were calibrated by provoking the crab to squeeze a calibrating device that had an output voltage directly proportional to the force being applied (see Boulding, 1984). Visual observations verified that only during the actual attack of a bivalve were the forces produced by the chela sufficient to deflect the pen of the recording apparatus.

Simulation of predation—live bivalves

The bivalves (*Protothaca staminea*) were collected from Wescott Bay and from Garrison Bay, San Juan Island, Washington. Only animals free of visible growth irregularities were used. Shell height (umbo to ventral margin), shell length (anterior to posterior), shell width (most distal point of each valve from the plane of the commissure, valve thickness (1 mm above the central ventral margin), and submerged shell weight (weight of live bivalve in seawater, correlation coefficient with shell weight in air = 0.9987, $P < 0.01$) were measured for each bivalve.

Force was applied to the shells through small brass columns bolted to the compression plates of a Monsanto tensometer. To insure that local stresses were proportional to applied loads, the area through which the force was transmitted to the shell was measured by placing carbon paper between the shell and the brass columns; variation in contact area was small ($\bar{X} = 18$ mm^2; SD = 1.8). For the static loading tests the tensometer was manually operated so that the force increased at 33 Ns^{-1} until failure occurred. The bivalves (shell lengths 20–60 mm) were oriented with the ventral margin down and the right and left brass columns touching the central region of the right and left valves, respectively, mimicking the crabs' "crushing outright" method of opening shells.

The usefulness of various shell measurements for predicting static strength was investigated using multiple linear regression with load at failure as the dependent variable and various combinations of the shell measurements as independent variables. All the variables were log-transformed to linearize the relationships.

To test whether bivalve shells fatigue significantly when loaded with a large load for a low number of cycles, bivalves were loaded with 80% of their static strength (as predicted from the regressions on the shell measurements) until they failed. Other bivalves were subjected to loads that had the maximum amplitude stepped up by 10% if no failure had occurred after 4 cycles at the same load.

Simulation of predation—cleaned valves

The valves of freshly killed *Protothaca staminea* were gently separated, cleaned, and glued to a wooden block with Devcon 5 minute epoxy so that the rim of the valve was in full contact with the block. Each valve was loaded at the center of its exterior surface using an Instron Universal testing machine (model TT-DM) at a head speed of 5 mm/min. This approximated the crabs' "crushing outright" method of opening shells as experienced by one valve of the shell. In order to keep the contact area constant and the contact surface smooth—so that the local stresses were proportional to the load applied—the force was applied through the molars of a cancrid crab chela glued to the upper plate of the Instron.

Each of 28 valves from 14 bivalves (shell length 52 to 58 mm) was loaded until failure occurred. The static strength of the left and right valves of the same shell were compared among shells using Analysis of Variance (ANOVA).

In order to predict static strength more accurately, the load at failure for one valve was used to predict the static strength of the other valve from the same shell. The second valve was then repeatedly loaded with a constant load of 70–100% of its predicted static strength using the automatic cycling control of the Instron. If no failure occurred within approximately 200 loading cycles the load was increased by 10% and the cyclic loading repeated. For some shells the load was increased and the cycling repeated four times before the shell failed. For three shells both valves were loaded cyclically with 80% of the predicted static strength for a shell with those dimensions from that site.

RESULTS

Crab predation behavior

Figure 1 shows the pulsed application of force by the chela of a *Cancer productus* to the shells of two bivalves. Failure of the shells does not necessarily occur during the greatest force pulse. Figure 1A (where failure probably occurred at F?) and other recordings (see Boulding, 1984) suggest that the magnitude of the force pulse at failure can be less than that of previous force pulses. These crabs load the shells at a rate of up to two force pulses per minute—one monitored crab continued to load a shell more than 265 times over a three day period before failure occurred—the number of

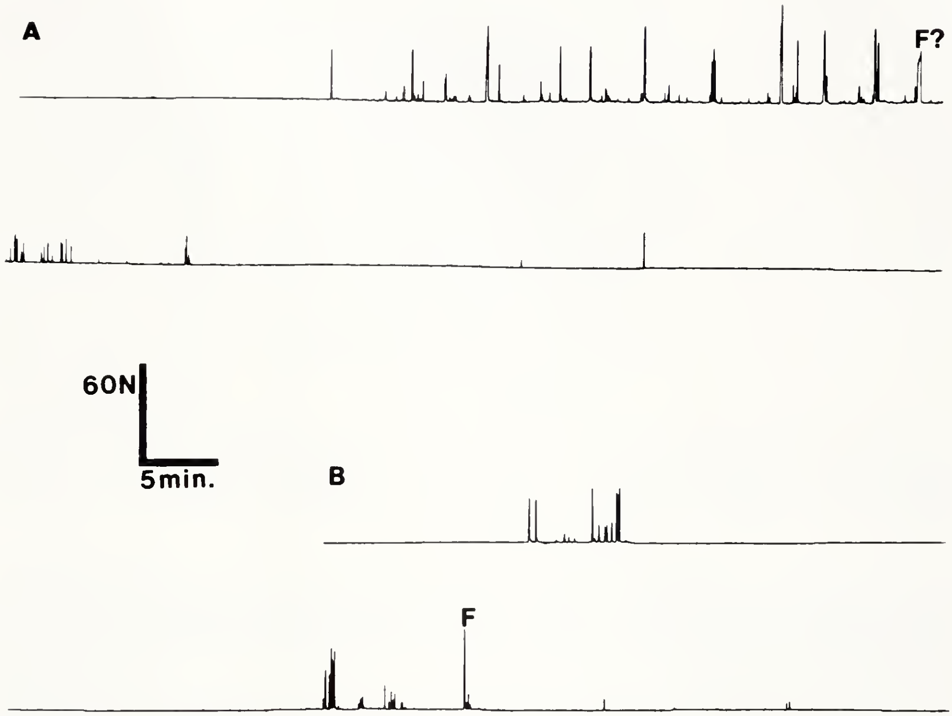

FIGURE 1. Continuous recordings (read from left to right) from a strain gauge glued directly to the chela of a female *Cancer productus* (carapace width 86 mm): in (A) which was not observed, the crab attacked a *Protothaca staminea* (thick-shelled morph; shell height 16 mm, shell length 18 mm) and in (B) which was directly observed, the same crab attacked a *P. staminea* (thick-shelled morph; shell height 14 mm, shell length 16 mm). In (B) the long interval between the two groups of force pulses occurred as the crab was disturbed by being photographed.

force pulses applied and the total handling time increased with the relative size of the bivalve (see also recordings in Boulding, 1984).

Simulation of predation—live bivalves

Although a wide size range of bivalves was used, a large part (>35%) of the variation in the static strength of the shells could not be explained by a multiple linear regression using shell height, shell length, and shell weight as the independent variables ($r^2 = 0.646$, $N = 30$, $P < 0.01$). If shell height alone was used to predict static strength the equation: static strength = 97.5∗ shell height was obtained ($r^2 = 0.401$, $N = 30$, $P < 0.01$).

This latter equation was not a good predictor of static strength for a second group of 30 bivalves from the same site; when these bivalves were repeatedly loaded with 80% of their predicted static strength, 16 out of the 30 failed on the first loading cycle. Of the 14 remaining shells, 6 continued to be loaded repeatedly with 80% of their predicted static strength while 8 were subjected to stepped loads that were increased by 10% if no failure occurred after 4 cycles. All 14 of these bivalves ultimately failed under loads less than or equal to loads that had been previously applied. Figure 2

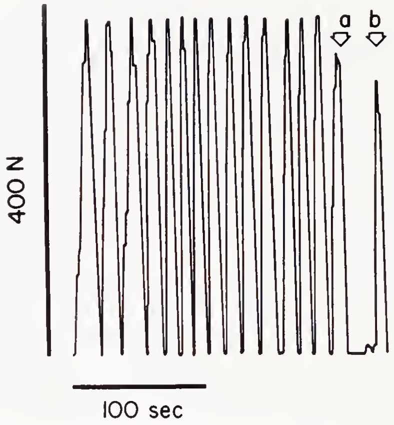

FIGURE 2. Recording of load *versus* time for a live bivalve loaded in the Monsanto tensometer (read from left to right). This shell was loaded 14 times before it cracked on the 15th cycle (a) and failed on the 16th (b).

shows a typical recording from a bivalve that was loaded with a subcritical load 14 times before it cracked on the 15th cycle and failed completely on the 16th.

Simulation of predation—cleaned valves

The static strengths of isolated valves from the same bivalve shell were more similar to each other than to the mean for all valves (ANOVA, N = 14, $P < 0.01$) even though the dimensions of all these shells were very similar (shell length 52 to 58 mm). There was no significant difference in the static strength of the right and left valves (sign test $P < 0.05$).

The fatigue life of two valves from the same shell could be appreciably different. For one shell, the right valve withstood 15 cycles while the left withstood 4 cycles of the same load. In another case the right valve failed after 261 cycles while the left withstood 579 cycles without failing. In a third case the right valve failed after 226 cycles while the left valve sustained 1666 cycles without failing.

In a group of 30 bivalves that had one valve loaded statically and one valve loaded

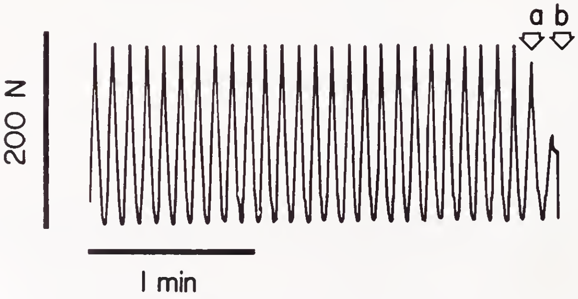

FIGURE 3. The final portion of a recording of load *versus* time for a valve loaded in the Instron (read from left to right). The shell failed after 143 cycles of a load of 176.4 N. The valve cracked at (a) and failed at (b).

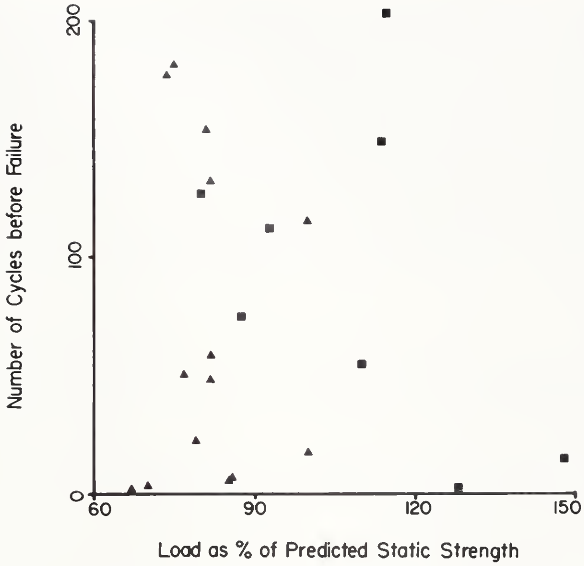

FIGURE 4. Number of cycles of loading withstood by single valves before failure *versus* the load as a percentage of their predicted static strength. The predicted strength used was that of the other valve from the same bivalve shell. The triangles represent valves that were repeatly loaded with a load that was constant in maximum amplitude. The squares represent valves where the load was constant for 200 cycles, then was increased by 10% if no failure had occurred. The number of cycles plotted for these latter (square) points is the number of cycles since the last increase in the maximum amplitude of the load.

cyclically (Fig. 3), 12 of the cyclically loaded valves failed on the first load, 10 withstood between 4 and 182 cycles before failing, and 8 withstood at least 200 cycles before failing and had their maximum loads increased by 10% up to four times.

There was no evident relationship between the load applied, measured as a percentage of the static strength of the other valve from the same shell, and the number of cycles withstood (Fig. 4). This was probably a reflection of the low precision with which it is possible to predict the static strength of whole shells. Only loads close to the actual static strength of the valve seemed to appreciably weaken the shell; the position of the stepped valves (squares) along the y-axis of Figure 4 relative to those loaded at a constant amplitude (triangles) suggests that the previous cycles of loading at lower maximum loadings had little effect on the number of cycles withstood at the greatest maximum loading.

DISCUSSION

Low cycle fatigue of bivalve shells

Literature on low cycle fatigue of structures made of brittle materials is scarce, and primarily concerns structures made of concrete. Concrete beams can show appreciable fatigue damage within a low number of cycles when loaded with more than 75% of their predicted static strength (J. G. MacGregor, pers. comm.). Low cycle fatigue has been reported for human leg bones (see Wainwright *et al.*, 1976) and for strips of pearl oyster nacre for loads above 60% of the predicted static strength (Currey and Brear, 1984).

The dependence of fatigue life (number of cycles withstood) on the magnitude of the loads applied is probably related to the accumulation of fatigue damage. Each time a brittle structure is loaded, the resulting strain can be divided into two components: an elastic component that is recoverable upon unloading, and a plastic component that is not recoverable and takes the form of microcracks. Under small loads, while the relationship between load and deformation remains linear, most of the strain is elastic although there will always be some plastic strain even if it is unmeasurable (Sandor, 1972). Under large loads where the load-deformation relationship is nonlinear, the plastic component becomes a substantial proportion of the total strain (Sandor, 1972). Some engineers hypothesize that the amount of plastic strain energy that a given structure can absorb is fixed, and that failure will occur when this limit is reached whether the limit occurs during a single loading with a large load or after many loading cycles with smaller loads (Lefebvre and Ellyin, 1984). Thus, the number of cycles of loading and unloading that can be withstood would be predicted to decrease exponentially rather than linearly with the increasing magnitude of the load applied.

Fatigue life will also depend on the stress concentrations caused by pre-existing cracks or other flaws (Broek, 1982). High contact stresses resulting from concentration of all the force generated by the chela muscles onto the small area of the shell in contact with the chela molars could hasten the development of fatigue damage. Things are further complicated because the important stresses in a bivalve shell loaded on the apices of its two valves by a crab are probably not just those from simple bending. In simple bending, the greatest tensile stresses would be immediately below the loading points on the inside of each valve and the greatest compressive stresses would occur immediately below the loading points on the outside of each valve. The domed shape of *Protothaca* could result in unidirectional tensile stress on the inside of the valves being replaced by a two dimensional (membrane) stress field, especially if the shell margins are restrained from splaying out by friction along the ventral margins (Timoshenko and Woinowsky-Kreiger, 1959), and thus increase the amount of force a domed shell can withstand relative to that which a flat shell could withstand.

The dependence of fatigue life on stress concentrations was one reason we used whole shells instead of standardized test strips cut from shells to test for susceptibility to low cycle fatigue; because the geometries of whole shells and test strips are different their patterns of force concentration are different too. In addition, microcracks can result from the machining of test strips (Currey and Kohn, 1976) which could confound the results of fatigue tests made with test strips. By not using test strips we avoided these complications at the cost of precision in our estimates of static strength.

We could not use a mechanical testing machine to satisfactorily mimic the "edge-chipping" method that crabs use to open bivalves too large to fit entirely within a chela, but we believe that fatigue damage may also be involved in the chip formation seen in this mode of attack. During "edge-chipping," the crab's chela slips ventrally on the shell as the crab squeezes its chela around the ventral margin of the shell. The net force at a given chela molar can be resolved into two vectors: a large compressive vector normal to the shell surface and a smaller shearing vector parallel to the shell surface. A theoretical analysis (Boresi *et al.*, 1978) suggests that if the coefficient of friction of the chela on the shell is greater than $^1/_{10}$, the region of greatest tensile stress will move from the inside of the valve to the outer valve surface. These contact stresses can be the most significant stresses in the structure—particularly when the surfaces do not remain in fixed contact—and would be expected to lead to a type of fatigue failure that takes the form of pitting (Boresi *et al.*, 1978). Thus the formation of chips at the ventral margin of a bivalve shell during "edge-chipping" may result from the repeated slipping of the contracting chela over this surface.

Bivalves are probably unable to repair microcracks out of reach of their mantle such as would occur on the outside surfaces of their shells; microcracks would thus be expected to accumulate on these regions and would make the shell especially vulnerable to loading regimes where the maximum tensile stresses are on these surfaces. Even when the maximum tensile stresses do not occur on the outer surfaces of the shell, the greater vulnerability of this layer is obvious; after loading and unloading a shell in the Instron many times we sometimes saw the outside layer crumbling away even though the inner layer was still intact.

Importance of repetitive loading behavior to crabs

Our results suggest that a crab's ability to open a bivalve shell is not simply a matter of whether the maximum force the crab can exert is greater than the static strength of the bivalve shell. Indirect force recordings from the crab's chela indicated that failure of prey shells could occur during force pulses less than or equal to those that had been applied previously. Live bivalves cyclically loaded in the tensometer with 80% of their predicted static strength and cleaned valves cyclically loaded in the Instron with 70–90% of their predicted static strength often failed in fewer than 200 loading cycles. This supports the hypothesis that bivalve shells are susceptible to low cycle fatigue if repeatedly loaded by crabs with a high proportion of the shells' static strength.

The results of the static loading investigations suggest that it would be difficult for crabs to predict the static strength of a bivalve's shell on the basis of its physical measurements. Shell height, shell length, and shell weight accounted for only 65% of the variation in static strength of these *Protothaca staminea* (shell length 20–60 mm); adding other variables such as valve thickness did not substantially increase the variation accounted for. Indeed, in a detailed study of scallop shells only 64% of a shell's tensile compliance could be predicted from the physical measurements of the shell such as thickness and corrugation amplitude (Pennington and Currey, 1984). We suspect that the remaining 35% or so of the variation in static strength is due primarily to historical factors such as wear or previous loading history. Indeed, the predicted fatigue life of standard concrete test specimens is usually given in probabilistic terms—cumulative probability of failure after a given number of loading cycles (Anonymous, 1974)—rather than as a simple function of the load applied. Our interpretation is supported by the relative similarity in the static strength of two valves from the same shell when compared with valves from other bivalves with similar physical measurements. Our results suggest that the fatigue life of a bivalve shell under a given loading regime would be even more difficult to predict than static strength unless the crab could somehow detect minute increases in compliance.

Given the unpredictable nature of the fatigue life of bivalve shells, it would seem at first a poor strategy for a crab to persist in squeezing a bivalve on the chance it might fail. However even if a crab can only exert a maximum of 70% of the force necessary to crush a given shell the microcracks propagated by the larger force pulses could be propagated by the smaller force pulses (Sandor, 1972). We will show that under certain circumstances it pays for crabs to persist in attacking a shell.

Crabs prefer small bivalve prey which they can open easily and rapidly (Boulding, 1984), and when crabs are abundant such prey may become scarce. By repeatedly loading shells, crabs greatly increase the size range of prey available to them. For example if a 60 mm male *Cancer productus* used only a single contraction of the chelar muscles, the largest *Protothaca staminea* (thick-shelled morph) opened was one of 11 mm shell length, whereas if the crab used repeated loading of the ventral margin it could open bivalves greater than 49 mm (E. G. Boulding, pers. obs.). Periods of low prey abundance could result in selection for crab behavior and morphology that increased the size range of prey accessible to the crabs. Very high selection coefficients have been documented for Darwin's finches when a drought markedly reduced seed availability and selective consumption by predators increased the average size and hardness of those seeds that remained (Boag and Grant, 1981).

Crabs have demonstrated sophisticated predatory behavior that involves prolonged attack with repeated loading of the same region of a mollusc shell. The tropical crab *Carpilius maculatus* has been observed to persistently apply force pulses to the same region of tropical gastropod shells (Edith Zipser, pers. comm.). *Carcinus maenas* has been observed to repeatedly apply its chela to same spot on a mussel (Elner, 1978) and has been observed to squeeze bivalves for more than 20 minutes (Elner and Hughes, 1978) at a rate of 10 pulses per minute (Elner, 1978), approximately 200 loading cycles. *Cancer productus* has been observed to persistently attack the same region of the ventral margin of a *Protothaca* shell even after the crab had put down the shell and picked it up again; we have recorded a crab of this species continuing to squeeze the shell at the rate of 1 to 2 pulses per minute for three days (albeit with some pauses). *C. productus* will sometimes alternately attack a bivalve and walk around the aquarium searching for other prey, and this sequence often continues until the crab successfully opens the bivalve (Boulding, 1984).

Although smaller bivalves are much more quickly opened and are preferred in the laboratory (Boulding, 1984), crabs may reduce their risk of predation by carrying a large prey item to a safe place and repeatedly loading it until it fails. Tropical crabs were often found under boulders or large chunks of corals in association with the shell fragments of massively thick shells which are known from laboratory observations to require prolonged attack (Zipser and Vermeij, 1978; E. G. Boulding, pers. obs.). The recovery location of characteristically chipped shell fragments of previously marked *Protothaca staminea* implied that crabs were transporting these large, difficult-to-open bivalves from the intertidal to subtidal eelgrass beds (Boulding and Hay, 1984) where the crabs were presumably safer from predators.

Thus there is evidence that crabs persistently attack bivalves and gastropods, and that the prey shell is progressively weakened by fatigue damage until it fails. If the cost of continuing the attack is low, the risk of leaving the refuge high, and alternative prey scarce, then crabs may benefit most by continuing the attack.

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THE USE OF MUCOUS TRAILS BY INTERTIDAL LIMPETS TO ENHANCE FOOD RESOURCES

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ABSTRACT

The mucous trails secreted by certain species of intertidal limpets serve as adhesive traps for the microalgae that are their primary food resources. The mucous trails of two solitary homing limpets, *Lottia gigantea* and *Collisella scabra,* also stimulate the growth of microalgae. In contrast, the mucous trails of an aggregating limpet, *Collisella digitalis,* and the carnivorous dog whelk, *Nucella emarginata,* do not stimulate microalgal growth. These results may be explained by differences in the behavior of these gastropod species. Homing species can capitalize on the production of a food enhancing mucus because they have restricted home ranges and retrace their own mucous trails.

The persistence time of mucus in the field varied with gastropod species, mucus type (*i.e.,* mucus produced by moving or stationary limpets), and tidal height. Field studies suggest mucous trails can, but may not always, persist long enough to allow algal enhancement.

Biochemical analyses suggest that the ability of mucous trails to trap microalgae adhesively is correlated with carbohydrate content. The ability of mucous trails to stimulate microalgal growth is correlated with higher total organic content of mucous secretions and an ability to stimulate bacterial growth. A bacterial film may be important in the stimulation effect.

INTRODUCTION

As a gastropod crawls, it secretes a thin mucous layer that couples muscular movement to the substrate, thus powering locomotion while also permitting adhesion (Denny, 1980a; Grenon and Walker, 1980). A significant portion of a snail's energy budget (7–26%) is devoted to pedal mucus production (Calow, 1974; Denny, 1980b) and pedal mucus production is associated with most gastropod activities (reviewed in Connor, 1983). Mucous trails function as chemical cues used to locate home sites, conspecifics, or established foraging routes (McFarlane, 1981). Pedal mucus also is involved in desiccation reduction (Wolcott, 1973), predator avoidance (Harrold, 1982), and home scar formation (Lindberg and Dwyer, 1983).

In this paper I elaborate on the evidence and mechanism for another function of limpet mucus, the involvement of mucus in the feeding ecology of limpets. Many species retrace and ingest mucous trails. These trails are adhesive and can trap microalgae that may serve as an additional food resource. In addition, certain species of homing limpets secrete mucous trails that stimulate the growth of the algal food spe-

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cies for later consumption, thus further supplementing the diet of the mucus producer (Connor and Quinn, 1984).

In a previous paper (Connor and Quinn, 1984) these phenomena were reported and explained in terms of the natural histories of the three limpet species studied. In the current study I extend the initial observations to evaluate the importance of a provendering ability under field conditions. First, to determine if algal enhancement is a unique property of the mucus produced by herbivores, the mucus of a carnivorous snail was examined. Second, the provendering abilities of pedal mucus were evaluated at a high and low intertidal site. The persistence of mucus at these sites also was monitored to determine whether mucous trails last long enough in the field for provendering effects to be significant. In addition to examining the importance of provendering abilities, this paper will focus on the potential mechanisms of microalgal biomass enhancement. First, the relationship between mucus type and bacterial growth rates was examined. A second approach was to determine the biochemical composition of the various mucus types and look for chemical components that varied among the species in ways corresponding to the species ranking patterns seen for microalgal adhesion, mucus persistence, or growth stimulation (microalgal and bacterial).

MATERIALS AND METHODS

Experimental animals

The provendering ability of mucous trails was examined in three species of acmaeid limpets found along the western coast of North America: *Lottia gigantea* Sowerby 1834 (hereafter referred to as *Lottia*), *Collisella scabra* (Gould) 1846, and *Collisella digitalis* (Rathke) 1833. All three are herbivores, grazing primarily on microalgae and encrusting algal forms. *Lottia* inhabits the high and mid-intertidal zones of exposed rocky coasts. It actively defends discrete territories from which it rarely ventures (Stimson, 1970, 1973; Sibley, 1982). It is a homing species, and characteristically returns to a home scar after all of its foraging activities.

Collisella scabra is a common inhabitant of the splash and upper intertidal zones. This limpet has been well studied, including its ability to home and form home scars (reviewed by Lindberg and Dwyer, 1983; Kunz and Connor, 1986). The ability of an individual *C. scabra* to retrace its own mucous trail in order to locate its home scar has been reported by Hewatt (1940); however, an individual does not have to retrace the outward trail to return home. Although the consistent return to a home scar increases limpet survival (Wells, 1980; Kunz and Connor, 1986), it also restricts the foraging range of *C. scabra.*

Collisella digitalis is another common inhabitant of the upper intertidal and splash zones. However, in contrast to both *Lottia* and *C. scabra, C. digitalis* does not form home scars. *Collisella digitalis* commonly forms large aggregations by following the mucous trails of conspecifics. Generally, *C. digitalis* does not home, and is more wide ranging in its foraging than either *Lottia* or *C. scabra* (Frank, 1964; Breen, 1972, 1973; Connor, 1983).

To help identify the functions of mucous secretions produced by herbivores, I also examined mucus function in a predatory gastropod, *Nucella emarginata* (hereafter referred to as *Nucella*). *Nucella* is a neogastropod that is found in the mid-intertidal zone and preys on barnacles and limpets (Abbott and Haderlie, 1980).

Animal maintenance

Experiments were conducted from 1981 to 1983 at the Bodega Marine Laboratory, Sonoma County, California. All organisms used in experiments were collected from sites on or adjacent to the Bodega Bay Reserve, except *Lottia* which is not found at Bodega Bay. *Lottia* was collected near Santa Barbara or on San Nicolas Island, California. All animals were maintained in aquaria coupled to a flow-through seawater system. All snails were used within 48 hours of collection, except specimens of *Lottia,* which were used for up to two months after collection.

Mucus collection

Mucus was collected by rinsing limpets in seawater and positioning them on sloping glass plates. Limpets were placed at the bottom of plates and removed after they crawled to the top. In order to initiate movement, limpets were sprayed with seawater to simulate the incoming tide. The mucus produced by stationary limpets (hereafter called stationary mucus) is different in physical and chemical properties from trail mucus (Connor, 1983). The central portion of the plate contained primarily trail mucus and this mucus was removed with a razor blade. The potential for contamination of trail mucus with stationary mucus was greater in *Lottia* and *C. scabra* than in *C. digitalis* because these homing species are not as mobile as *C. digitalis,* and they often stopped moving. *Nucella* does not produce a distinct stationary mucus.

Mucus as an adhesive trap for microalgae

To determine differences in the adhesion of microalgae to the mucous trails of different gastropod species, trail mucus was collected from each species and spread evenly over one side of 12 Millipore filters (5 cm diameter). The mucus-coated filters were attached to Plexiglas sheets and suspended in battery jars filled with circulating microalgae cultures. Cultures contained a mixture of microalgal species (*Fragilaria, Navicula, Coscinodiscus, Nitzschia, Achnanthes, Skeletonema, Synedra, Melosira,* and two unidentified single-celled species of green algae) collected from the same field sites where limpets were collected. Microalgae were collected using the scrubbing technique of Nicotri (1974). Filters were exposed to the microalgal cultures for 18 hours in the dark (to minimize microalgal growth), after which the amount of microalgae adhering to the filters was estimated spectrophotometrically using the chlorophyll analysis technique of Strickland and Parsons (1968). Filters not coated with mucus also were placed in algal cultures to serve as a control. As an additional control, mucus-coated filters were placed in jars containing filtered seawater.

Mucus as an algal growth stimulant

To determine whether mucus acts as a microalgal growth stimulant, 1 ml aliquots of the microalgal culture used in algal adhesion experiments were added to mucus-coated filters (10 filters per species) and uncoated control filters placed within Plexiglas wells (5 cm diameter). The filters were kept wet and incubated in continuous light (Gro-lights, General Electric). The filters were removed after seven days and algal biomass was determined as in the adhesion experiment. Preliminary experiments indicated that six days were required before a significant increase in microalgae was detectable. As an additional control, mucus-coated filters were placed in Plexiglas wells containing filtered seawater.

Field experiments

Field experiments were conducted to determine the relevance of laboratory results to field conditions. Millipore filters coated with mucus and uncoated filters as controls (sample size ranged from 9 to 13 filters per treatment) were attached to Plexiglas panels and anchored vertically at the mouth of a semi-protected surge channel at +1.7 m and +.15 m above mean lower low water. The high site represents a typical habitat for all three limpet species. Macroscopic algae are rare at this level, but both *Collisella* species and barnacles (*Balanus glandula*) are abundant. The lower site was situated at a level below that normally inhabited by the three limpet species, although juveniles of all species (<8 mm) are found here within a highly diverse community of invertebrates and macroscopic algae.

The amount of microalgae adhering to the filters was determined after one day (high intertidal site only) and after seven days (both sites). The one-day experiment minimized time for algal growth and thus gave an estimate of the amount of algae adhesively trapped. The seven-day experiment measured the combined effects of adhesion and stimulation.

Mucus persistence

The field experiments combined the effects of mucous trails acting as adhesive traps and mucus acting as a microalgal growth stimulant. To determine whether mucous trails last long enough in the field for the stimulation effect to be significant (*i.e.,* the seven days required in laboratory tests), a series of laboratory and field experiments examined the persistence of mucus.

Field tests. To measure mucus persistence in the field, individual gastropods were allowed to crawl over Plexiglas sheets for eight hours. All mucous secretions were clearly visible after the Plexiglas sheets were dipped in a suspension of carbon particles (decolorizing carbon) and rinsed in seawater. Trails and stationary mucus were outlined on the undersides of the Plexiglas sheets with a waterproof pen. The sheets were mounted on boards positioned at the same sites used in the field algal adhesion and stimulation experiments. A grid system was marked on each sheet and the specific types of mucus present within each grid cell (5 × 5 cm, n = 200 cells per tidal height) were noted. Mucus persistence was monitored each day by watching for the complete disappearance of the attached carbon particles from each grid cell. Mucus breakdown was assumed to be complete after all the carbon particles were in suspension.

Laboratory tests. To determine the persistence of mucus in the absence of wave action and variable tidal submergence, the technique of Calow (1979) was employed. Plastic petri dishes were filled with seawater and a single snail was placed in each dish for eight hours. Both mucous trails and stationary mucus were then coated with carbon particles. Both mucus types were outlined on the undersides of the dishes with a waterproof pen. Dishes were refilled with seawater and maintained at 11°C. Each day, dishes were swirled and mucus persistence evaluated.

Mucus as a bacterial growth stimulant

Bacteria were collected by scrubbing intertidal rock surfaces and dipping the scrub brush into sterile seawater. The resulting water samples were filtered (Whatman #2) to remove rock particles and microalgae, and vortexed to suspend the bacteria prior to inoculation.

Five mucus samples (50 mg wet weight) of each gastropod species were sterilized under U.V. light for 16 hours. Each sample was added to a sterile culture tube containing 3.5 ml sterile TRIS buffer (pH 7.5) and 3.4 ml sterile filtered seawater (0.22 micron Millipore filter). To four of the five samples for each species, a 0.1 ml aliquot of bacteria suspension was added to each tube; sterile seawater was added as a control to the remaining tube. An additional control set of four test tubes contained bacteria, but no mucus. Tubes were sealed and placed in a test tube roller. After 44 hours, bacteria growth was determined qualitatively by recording the optical density of each test tube at 560 nm (Baumann and Baumann, 1980; Schwartzkoff *et al.*, 1983).

Biochemical characterization of mucus

Trail mucus was collected and immediately lyophilized. Because all analyses were conducted with fresh freeze-dried mucus, the data include values for any material associated with the protein and carbohydrate components (as noted in Grenon and Walker, 1980). Values reported for each biochemical assay represent the average of three replicate samples.

Water content. Water content was determined from the weight difference between fresh and freeze-dried mucus. Sample weights were determined on a Cahn microbalance.

Inorganic content. Pre-weighed aliquots of freeze-dried mucus were ashed in a muffle furnace for five hours at 550°C. The remaining inorganic residue content was calculated from the weight difference between pre- and post-ashed mucus samples.

Protein. The total protein content was determined using the micro Bio-Rad assay technique (Coomassie blue). Approximately 3 mg mucus (dry weight) were homogenized in 80% ethanol and centrifuged at $10{,}000 \times g$ for 30 minutes. The pellets were dissolved in hot 1.0 N NaOH and then assayed spectrophotometrically after the addition of Coomassie blue dye (Bradford, 1976). Bovine serum albumin (Bio-Rad) was used as a standard.

Carbohydrate. Ethanol-soluble and insoluble carbohydrate levels of 3 mg mucus (dry weight) were measured by the micro-anthrone assay (Keleti and Lederer, 1973) using glucose as the standard.

Lipid. Lipid levels were determined by gravimetric measurement of lipid extracts. Approximately 3 mg of mucus was homogenized with 0.5 ml of distilled water. One ml of a chloroform-methanol solution (2:1) was added to the mucous homogenate which was then probe-sonicated (Biosonik III) for 30 seconds. Samples were centrifuged on a clinical centrifuge for two minutes at 2000 rpm to separate the lipid-containing chloroform phase from the homogenate. After pipetting this layer into tared pans, the chloroform was evaporated and lipid weights were determined.

Free amino acids. Concentrations of free amino acids were determined using the fluorescamine assay for primary amines (North, 1975). A mucous sample (0.5 mg) was added to 1.0 ml of .2 M sodium borate buffer (pH 9.0). While vortexing this mixture, 0.5 ml of fluorescamine (15 mg/100 ml acetone), followed by 2 ml of distilled water, were added to the sample. The fluorescence of each sample was determined on a spectrofluorometer with the excitation wavelength set at 390 nm and the emission wavelength set at 480 nm. Glycine was used as the standard.

Statistical analyses

Most experimental results were analyzed for interspecific differences in pedal mucus properties by an analysis of variance followed by mean separation tests (Student-

Newman-Keuls multiple range test). Adhesion, stimulation, laboratory persistence, and bacteria growth data were log-transformed before analysis. Biochemical data were angular (arcsine) transformed before analysis. Means and standard errors presented in tables are untransformed values. At the end of 30 days, when the mucus field persistence experiment was terminated, there were still patches of mucus on the Plexiglas sheet located at the high intertidal site. Because the actual persistence time of these patches could not be estimated, nonparametric techniques using ranked persistence values were used to compare mucus types within the higher site and to compare the differences in persistence between the two tidal heights. The Kruskal-Wallis test and a nonparametric multiple comparison technique (Zar, 1974) were used in these comparisons.

RESULTS

Mucus as an adhesive trap

In the laboratory studies, the amount of microalgae adhering to pedal mucus varied significantly among the different gastropod species. *Lottia, Nucella,* and *C. scabra* produced trails that were not significantly different from each other ($P > .05$) in terms of adhesion, but were more adhesive ($P < .05$) than the trails of *C. digitalis.* The amount of algae adhering to *C. digitalis* trails was not significantly different ($P > .05$) from the amount of microalgae adhering to the mucus-free surfaces of the control filters (Table I). No chlorophyll was detected on mucus-coated filters placed in battery jars containing algal-free seawater.

Mucus as an algal growth stimulant

In the laboratory study, chlorophyll analyses indicated a significantly higher ($P < .05$) microalgal biomass on the trail mucus of *Lottia* and *C. scabra* than on the mucus of the other species. In addition, the microalgal biomass levels on *Lottia* trails were higher ($P < .05$) than on *C. scabra* trails. Algal biomass on *Nucella* and *C. digitalis* samples was not significantly different ($P > .05$) from levels measured for the mucus-free control filters (Table I). No chlorophyll was detected on mucus-coated filters that had been placed in algal-free seawater. Only the two solitary homing species, *Lottia* and *C. scabra,* produce a mucus with significant algal growth enhancement properties.

Field experiments

In the one-day field experiment it was assumed that the differences in the amounts of microalgal biomass attached to the filters were due primarily to differences in mucus adhesiveness. The amount of microalgae adhering to the mucous trails of all four gastropod species was significantly higher ($P < .05$) than the amount of microalgae adhering to the mucus-free surfaces of the control filters (Table I). The overall trend suggests that *Lottia* and *C. scabra* have stickier mucous trails than *C. digitalis,* although the difference between *C. scabra* and *C. digitalis* was not significant ($P > .05$). In contrast to the results of the laboratory adhesion experiments, there was no difference ($P > .05$) between *C. digitalis* and *Nucella* trails in attached microalgae (Table I).

The seven-day field experiment was conducted at both high and low intertidal heights. Filters coated with mucus from all three limpet species had greater microalgal

TABLE I

Effects [illegible] mucus on adhesion and growth in microalgae (milligrams of chlorophyll per 25 cm²) and growth in bacteria (optical density at 560 nm)

Experiment	*Lottia gigantea* mucus	*Collisella scabra* mucus	*Collisella digitalis* mucus	*Nucella emarginata* mucus	Control (no mucus)	Control (filtered seawater)
			Laboratory			
MICROALGAE						
Adhesion (18 hours)	78.3 ± 9.7^a (12)	67.5 ± 5.5^a (12)	26.5 ± 5.8^b (12)	74.6 ± 9.9^a (12)	18.2 ± 3.6^b (12)	0.0^c (8)
Growth (7 days)	72.9 ± 5.7^a (14)	56.2 ± 5.8^b (10)	35.6 ± 2.8^c (10)	39.9 ± 3.2^c (10)	39.2 ± 2.8^c (10)	0.0^d (8)
			Field sites			
Adhesion (1 day at high intertidal site)	26.2 ± 2.8^a (9)	14.5 ± 1.5^b (10)	9.4 ± 2.7^{b,c} (9)	9.0 ± 2.0^c (10)	5.0 ± 1.1^d (9)	
Adhesion and growth (7 days)						
High intertidal site	3.2 ± 0.4^a (12)	3.1 ± 0.5^a (11)	2.2 ± 0.2^{a,b} (10)	—	1.8 ± 0.3^b (9)	
Low intertidal site	15.8 ± 1.6^a (13)	9.8 ± 1.3^b (13)	15.1 ± 1.8^a (12)	—	10.9 ± 0.9^b (10)	
BACTERIA					(bacteria and no mucus)	(mucus and sterile seawater)
Growth (44 hours)	0.071 ± 0.005^{a,b} (4)	0.143 ± 0.054^a (4)	0.030 ± 0.006^b (4) (trail mucus) 0.080 ± 0.024^{a,b} (4) (stationary mucus)	0.042 ± 0.008^{a,b} (4)	0.005 ± 0.001^c (4)	0.0016 ± 0.0005^d (5)

Values are untransformed means ± standard errors. Analysis of variance and mean separation by the Student-Newman-Keuls multiple range test were performed on logarithmically transformed data. For interspecific comparisons in each experiment, values with different superscripts are significantly different ($P < 0.05$). Sample size is indicated in parentheses.

TABLE II

Persistence time (days) of trail and stationary pedal mucus

	Lottia gigantea mucus		*Collisella scabra* mucus		*Collisella digitalis* mucus		*Nucella emarginata* mucus
Experiment	Trail	Stationary	Trail	Stationary	Trail	Stationary	Trail
Laboratory	11.2 ± 1.0^{a} (18)	7.3 ± 0.4^{b} (33)	17.8 ± 0.6^{c} (11)	9.1 ± 0.6^{d} (29)	18.8 ± 1.4^{c} (16)	6.2 ± 0.4^{b} (25)	20.1 ± 1.3^{c} (20)
High intertidal site	15.2 ± 1.4^{a} (30)	23.5 ± 1.5^{b} (36)	10.3 ± 1.8^{c} (18)	16.8 ± 2.3^{a} (16)	10.3 ± 1.1^{c} (29)	19.8 ± 2.7^{d} (16)	12.9 ± 1.6^{c} (35)
Low intertidal site	$5.0 \pm 0.2^{a,b}$ (34)	$5.0 \pm 0.4^{a,b}$ (36)	4.0 ± 0.3^{a} (17)	7.5 ± 0.7^{c} (13)	$5.5 \pm 0.3^{b,d}$ (31)	$6.8 \pm 0.4^{c,d}$ (12)	$6.6 \pm 0.4^{c,d}$ (33)

Values are means ± standard errors. For interspecific comparisons in each experiment, values with different superscripts are significantly different ($P < 0.05$). Sample size is indicated in parentheses.

biomass than the mucus-free control filters, although significant differences ($P < .05$) were detected only for *Lottia* and *C. scabra.* The species ranking pattern of the amount of microalgal growth enhancement seen in the laboratory was reproduced at the high intertidal site. As before, there was a trend with *Lottia* having the highest microalgal biomass levels, followed by *C. scabra,* and then *C. digitalis* (Table I).

The results from the low intertidal zone were different from those obtained in both the high site and from the laboratory experiments. First, compared to the high site, there were significantly higher ($P < .05$) levels of microalgal biomass across all treatments. Second, microalgal biomass levels were higher ($P < .05$) on *Lottia* and *C. digitalis* mucus samples than on filters coated with *C. scabra* mucus or uncoated control filters (Table I). The levels of microalgal biomass for all treatments in the 1-day experiment were greater than biomass levels in the 7-day experiment.

Mucus persistence

Field experiments. The number of days that mucus persisted varied with tidal height, gastropod species, and mucus type (*i.e.,* trail or stationary mucus) (Table II). Both stationary and trail mucus lasted longer ($P < .05$) at the high site for all species. Considering persistence results from just the high site, the median persistence time for the trails of all species is at least seven days; presumably sufficient time to effectively stimulate microalgal growth. The trails of *Lottia* lasted significantly longer ($P < .05$) than the trails of the other species, none of which were significantly different ($P > .05$) from each other in persistence time. The stationary mucus produced by all three limpet species lasted significantly longer ($P < .05$) than their trails. The stationary mucus produced by *Lottia* lasted significantly longer ($P < .05$) than the stationary mucus produced by the other two limpet species.

At the low intertidal site, trail persistence times ranged from an average of 4.0 days for *C. scabra* to 6.6 days for *Nucella.* Stationary mucus persistence ranged from an average of 5.0 days in *Lottia* to 7.5 days in *C. scabra. Collisella scabra* trails disappeared the most rapidly, but not significantly more so than the trails and stationary mucus of *Lottia. Lottia* stationary mucus lasted longer than any other mucus type at the high intertidal site, but was one of the first mucus types to disappear at the low intertidal site (Table II).

Table III

Summary of the biochemical composition of mucous trails

	Lottia	*C. scabra*	*C. digitalis*	*Nucella*	Significance
Proportion of dry weight:					
Inorganics	.430 ± .020	.470 ± .015	.450 ± .025	.757 ± .013	N SDL
Lipids	.003 ± .003	.004 ± .001	.008 ± .005	.002 ± .002	DSLN
Carbohydrates	.184 ± .002	.171 ± .009	.081 ± .013	.254 ± .035	NLS D
Proteins	.368 ± .075	.362 ± .024	.297 ± .039	.013 ± .008	LSD N
Free amino acids	.011 ± .002	.005 ± .000	.002 ± .000	.005 ± .000	L SN D
Total	.996	1.012	.838	1.031	
Dry weight/wet weight	.065 ± .009	.107 ± .009	.094 ± .006	.186 ± .005	N SD L

Values are means ± 1 standard error of three replicate samples. The last column summarizes interspecific rankings for each chemical component; letters that are underscored by the same line indicate species whose mucus is not significantly different in terms of the specific component (N = *Nucella emarginata*; S = *Collisella scabra*; D = *Collisella digitalis*; L = *Lottia gigantea*).

Laboratory experiments. Species rankings of mucus persistence time in the laboratory were opposite from persistence times in the field. In the laboratory, trail mucus was more persistent ($P < .05$) than stationary mucus. *Lottia* stationary mucus disappeared more rapidly than any other mucus type, whereas in the field *Lottia* stationary mucus was the most persistent (Table II).

Bacterial growth

The high variance and small sample size (n = 4) in this experiment precluded a statistically clear ranking of the mucus types in their effect on bacterial growth rates (Table I). Examining the overall trends, however, two points are evident. First, the trails of the homing species (*Lottia* and *C. scabra*) had higher bacterial growth rates than those of *C. digitalis* and *Nucella*. The difference between *C. scabra* and *C. digitalis* is significant ($P < .05$). Second, mucus produced by stationary *C. digitalis* individuals promoted bacterial growth better than *C. digitalis* trail mucus. The correspondence among laboratory persistence studies, microalgal stimulation experiments, and bacterial growth studies indicates a positive correlation between the ability to enhance microalgal growth and the ability to stimulate bacterial growth.

Biochemical characterization

The results of the biochemical determinations are summarized in Table III. The pedal mucous secretions of all four gastropod species consisted primarily of water, ranging from an average of 81.4% water for *Nucella* to 93.5% water for *Lottia*. The difference in water content among species appears small; however, evaluating water content as grams of water to gram dry weight of mucus reveals that *Nucella* mucus contained 4.38 g water/g dry wt, while *Lottia* mucus consisted of 14.38 g water/g dry wt—almost 3.3 times as much water. The mucus of *C. scabra* (8.35 g water/g dry wt) and *C. digitalis* (9.64 g water/g dry wt) contained an intermediate amount of water.

The amounts of inorganics present in pedal mucus were not significantly different among the three limpet species, and ranged from 43% of dry weight in *Lottia* to 47% of dry weight in *C. scabra*. The mucus from the carnivore, *Nucella*, contained a substantially higher proportion of inorganics (75% dry weight).

In all species, the lipid levels in pedal mucus were consistently less than 1% of the total dry weight.

Proteins and carbohydrates are the major organic components of pedal mucus. The carbohydrate concentrations in *Lottia* mucus (18.4% dry weight) and *C. scabra* mucus (17.1% dry weight) are higher ($P < .05$) than in *C. digitalis* mucus (8.1% dry weight). There was a non-significant trend in protein content among the three limpet species, with the protein levels in *C. digitalis* mucus (29.7% dry weight) somewhat lower than the protein content of *Lottia* (36.8% dry weight) and *C. scabra* (36.2% dry weight). The mucus of *Nucella* is clearly different from limpet mucus; its carbohydrate content is higher (25.4% dry weight) and its protein level (1.3% dry weight) is significantly lower ($P < .05$) than levels in limpet mucus.

The concentration of free amino acids in pedal mucus were higher in *Lottia* followed by *C. scabra,* and *Nucella,* and then by *C. digitalis.*

In summarizing the biochemistry results, it should be noted that on a dry weight basis, the mucus of homing limpet species (*Lottia* and *C. scabra*) contained more carbohydrate and protein than that of the non-homing, aggregating limpet, *C. digitalis.* This suggests that the mucus produced by *Lottia* and *C. scabra* is energetically more expensive to produce than the mucus of *C. digitalis.* Also, the mucus produced by the carnivorous *Nucella* was quantitatively different in several biochemical components from the mucus produced by the herbivorous limpets.

Discussion

The sticky trails of all the investigated gastropod species effectively entrapped microalgae in the field. Because this ability was found in all the gastropods examined (not only the herbivores), it may be a side-effect of producing a mucus that is effective in allowing the animal to adhere to the substratum.

The ability of mucous trails to stimulate microalgal growth was seen only in the homing limpet species, *Lottia* and *C. scabra.* It is not surprising that *Nucella* does not produce trails that stimulate algal growth; it is a carnivore and would not benefit from this ability. However, herbivorous *C. digitalis* does not produce a provendering mucous trail. Differences in the ability of mucus to stimulate the growth of microalgae may be understood by examining interspecific differences in natural history and mucus energetic cost. The explanation, presented in detail elsewhere (Connor and Quinn, 1984), is essentially the following. Both *Lottia* and *C. scabra* invest more energy in mucus production than *C. digitalis,* but this high cost is defrayed by the enhancement of food levels. In order for this type of strategy to be effective, a species needs to be the primary consumer of its own high cost trails with their entrapped microalgae. Thus, there must be a separation of individuals and the mucus producer should remain in the area to allow retracing of its trails. Both *Lottia* and *C. scabra* meet these two requirements. Both maintain home scars, have restricted ranges, retrace their own mucous trails, and behave aggressively when encountering other limpets (Wright, 1977; Sutherland, 1970). In contrast, *C. digitalis* forms aggregations and individuals frequently follow trails of conspecifics. Such aggregations and a relatively unrestricted home range should prohibit a limpet from using the trophic benefits derived from a high cost mucus.

In addition to having smaller home ranges, both *Lottia* and *C. scabra* exhibit an interesting behavior which may be related to the possession of a provendering mucus. When these limpets move out of their home scars, they slowly circle the home scar by rotating their bodies back and forth. Usually a considerable amount of mucus is laid down on the rock near the home scar before the limpets slowly move away to forage. When grazing occurs near the scar, it usually is done during the return trip to the home scar. This behavioral sequence would be expected to promote algal enhancement near the home site. In contrast, when the non-homing limpet species, *C. digitalis,* starts to forage, it will move away from its resting place in a straight line; there is no behavior resulting in extra mucus deposition and *C. digitalis* generally does not forage near its resting place.

Mucus persistence

The field experiments indicate that mucus appears to last long enough in the high intertidal zone for the stimulation of microalgal growth to occur. Field persistence values may even be conservative because mucus may not last as long on Plexiglas as it lasts on a more porous surface, such as the actual rock surface. However, it also should be noted that in the laboratory, six to seven days were required to detect significant differences in algal growth under conditions of constant illumination and submergence. Although it can not be assumed that, as in the laboratory, only seven days are required in the field for significant algal growth enhancement by mucous trails, Sechler and Gundersen (1973) report that diatoms begin to show "active growth" on glass surfaces after five days of exposure to field conditions. In addition, the grazing patterns of both homing species suggest that seven days is a reasonable estimate of minimum useful persistence time. Both *Lottia* and *C. scabra* generally forage over different sections of their home ranges during each foraging period. Although there is considerable variation in foraging behavior, these limpets generally do not regraze an area for at least four to seven days.

Low mucus persistence time in the laboratory correlates positively with high rates of bacterial growth, supporting the conclusion that mucus disappearance in the laboratory is due to bacterial decomposition. Because certain types of mucus are more susceptible to bacteria growth (*e.g.,* the stationary mucus and the trails of homing limpet species), it would seem reasonable to expect that these mucous secretions should break down faster under field conditions. In contrast to this expectation, the carbon particles used to mark mucus lasted longer on bacteria-enhancing mucus in the field.

This apparent contradiction may be explained by differences in the design of the laboratory and field experiments. In the laboratory, as mucus was broken down, the attached carbon particles immediately went into suspension because secretions were submerged continuously. In the field, at the high intertidal site, mucus was submerged for a maximum of four hours daily. Thus, as mucus was broken down by bacterial action, the particles were not immediately removed. The mucous film may have been replaced with a microbial film, and the carbon particles remained attached to this newly created film. The persistence of the carbon marker may not mean that limpet mucus was still present, but that some sort of adhesive film, replacing the limpet mucous film, had been produced by bacteria. Most marine bacteria produce an extracellular mucopolysaccharide while simultaneously decomposing existent organic films (Corpe, 1973; Sechler and Gundersen, 1973).

Mucus, or at least the carbon particles marking mucous trails, disappeared from the low intertidal site more rapidly than from the high intertidal site. This is probably because of the increased submergence time at the lower site. Increased submergence provides more time for sand scour and mechanical breakdown of mucus by wave action. Also, as indicated by the laboratory persistence experiments, increased submergence times at the lower site would reduce the chance that carbon particles would remain attached to any developing microbial film. It is not known if mucus lasts long enough at this level for algal growth stimulation to occur, even though the algal biomass attached to all experimental and control surfaces was higher at the lower intertidal site. Microalgal biomass levels might be expected to be higher at the lower site because microalgal growth rates increase with decreasing tidal height (Castenholz, 1961; Nicotri, 1974).

At the low intertidal site, filters coated with the mucous trails from *C. scabra* had significantly less microalgae attached to them than those of the other two limpet species. This may reflect the quick disappearance of *C. scabra* trails at this site. *Collisella scabra* trails disappeared more quickly than any of the other limpet species, although the difference between *C. scabra* and *Lottia* was not significant. The reason for the faster disappearance of *C. scabra* trail mucus at the low site is not known, but it is interesting in light of the behavior of the limpets living at this tidal height. Individual *C. scabra* larvae settle throughout the intertidal region and juveniles (<8 mm) may be found at low intertidal sites. In the field, juveniles do not home (Hewatt, 1940), perhaps because mucous trails at lower intertidal levels do not persist long enough to enable homing. However, juvenile *C. scabra* (1–2 mm) have been observed to home under laboratory conditions (Charles Baxter, Hopkins Marine Station, Pacific Grove, California).

Potential mechanisms of algal enhancement

Algal growth enhancement is assumed because of the differences in chlorophyll levels found for the different types of mucus. The specific microalgal changes responsible for these differences in chlorophyll biomass are not known. Mucous secretions may increase the growth rates or reproductive rates of one or more microalgal species. Because most of the species in the cultures were diatoms, the biomass increases were probably due to an increase in the number of individuals rather than an increase in the size of existing individuals. Many diatom species do not increase in size following asexual reproduction and cell size is continually reduced (in a one-cell line) during successive bouts of asexual reproduction (Bold and Wynne, 1978).

Consistency among results of laboratory persistence studies, microalgal stimulation experiments, and bacterial growth studies indicates a positive correlation between the ability to enhance microalgal growth and the ability to stimulate bacterial growth. There are several possible explanations for the stimulation of both bacterial and algal growth by the mucous trails of homing limpet species. First, it is possible that bacterial and algal growth are stimulated by separate chemical components, or that a single component simultaneously stimulates the growth of both. Several species of littoral pennate diatoms, including *Navicula* and *Nitzschia*, are capable of heterotrophic growth when supplied with sources of carbon and nitrogen (Lewin, 1953; reviewed by Hellebust and Lewin, 1977); mucus could serve as such an organic substrate. Marine bacterial growth also is limited by the amount of organic carbon present in seawater (Jannasch, 1967; Sieburth, 1979), so mucus could significantly enhance bacterial growth.

Bacteria have shorter generation times than diatoms, and thus an increase in bacteria is detected sooner; however, the ability of mucus to enhance bacterial growth, and then algal growth, suggests that bacteria may be involved in the process of stimulating algal growth. The idea of a bacterial film facilitating the growth of microalgae is well established. Bacteria produce an extracellular mucous film that is a prerequisite for the attachment and growth of microalgae (Corpe, 1973; Sechler and Gundersen, 1973; Young and Mitchell, 1973; Haines and Guillard, 1974; Sieburth, 1979). Corpe (1973) and Marshall *et al.* (1971) suggest that this mucous film is produced to bind bacteria to the substrate. However, considerable amounts of metabolic residues accumulating within this microbial film provide adsorbed, but non-developing, diatoms with an enriched substrate for growth (Sechler and Gundersen, 1973). Corpe (1973) found that the microbial film is a polyanionic carbohydrate with the ability to complex both metal ions and aggregate particulate materials. The breakdown of mucus by bacterial enzymes would be expected to make the mucous surface a rich source of nutrients and an ideal substrate for further biological activity such as microalgal growth (Corpe, 1973; Dring, 1982). Bacteria may provide microalgae with a limiting nutrient, for example, vitamin B-12 (O'Kelley, 1974; Provasoli and Carlucci, 1974). Alternatively, bacterial decomposition of limpet mucus might make the nutritional components of the mucus readily available for algal use. The intermediary role played by bacteria seems likely in the present study if the persistence of the carbon marker on mucus at the high intertidal field site was due to the replacement of limpet mucous trails by bacterial films.

Regardless of whether bacteria are necessary precursors for microalgal growth enhancement, there must be some differences in the mucus of the homing limpet species that are responsible for the observed provendering action. The biochemical analysis provides some possible explanations. The significant ability of *Nucella* mucus to entrap algae, but not stimulate microalgal growth, suggests that two distinct mechanisms are responsible for the adhesion and stimulation effects.

The ability of mucous secretions to adhesively attach microalgae is correlated with mucus carbohydrate content. That is, *Lottia, C. scabra,* and *Nucella* all produce trails that are stickier than *C. digitalis* in terms of algal adhesion rates and the mucus produced by *Lottia, C. scabra,* and *Nucella* also contains significantly more carbohydrate than the mucus of *C. digitalis.* There are polyanionic carbohydrate residues in gastropod pedal mucus (Denny, 1979). If an increase in carbohydrate content results in an increase in these polyanionic residues, an increased attraction of charged particles to pedal mucus might be expected. However, changing the concentration of a polymer may change it from being an adhesive to a dispersant (Hermans, 1983). Thus an adhesive role can not be assigned to a secretory product strictly on the basis of its chemical characteristics (Hermans, 1983). The chemical analysis presented here is meant to be preliminary; much more work is needed to define the chemical composition of pedal mucus and the relationship between ionic interactions and adhesion.

The ability of homing limpet species to produce algal growth stimulating trails is not related easily to the concentration of any specific component. The high carbohydrate content of the non-stimulating mucus of *Nucella* suggests that carbohydrate concentration alone cannot be responsible for algal growth enhancement effects. Protein levels are higher in the mucus of homing species, but this difference is not significant, again suggesting that this component may not be responsible for algal growth enhancement. On a dry weight basis, the mucus of homing limpet species contains more organics than non-homing species. This difference among mucus types may be responsible for species differences in microalgal stimulation. Both bacteria (Jannasch, 1967) and diatoms (Lewin, 1953) can grow more rapidly when the concentrations of certain organic substrates are increased. The inability of the mucus of *Nucella* and *C. digitalis* to significantly enhance microalgal growth may be due to relatively low amounts of organics in their mucus. However, all types of mucus contain organics, so even gastropods with relatively low amounts of organics might be expected to increase microalgal growth, but this was not observed. This suggests that some other component contained only in the mucus of homing limpets is involved.

Comparison of mucus composition on a dry weight basis may be misleading because of the significant species differences in mucus dry weight/wet weight. These differences suggest that it is more appropriate to look for chemical components that vary in proportion wet weight in ways corresponding to the patterns seen for microalgal adhesion and stimulation. However, this assumes that for all gastropod species a given weight of mucus covers a constant area of substrate, and this often is not true (Connor, 1983). Also, because the wet weight of mucus depends on the conditions of collection (Connor, unpub. data), dry weight comparisons probably are more accurate than wet weight comparisons.

Environmental variability

Another important consideration is the type of environment a species typically inhabits. Mucus may not last long enough in low intertidal sites or in sites of high wave energy and scour to effectively enhance food resources, although homing species do occur in these environments. It is noteworthy that *Lottia* does not exhibit territoriality in areas of high sand scour (Betsy Steele, University of California, Santa Cruz).

The differences between and within laboratory and field results obtained during this study emphasize the potential importance of environmental conditions on the various properties of mucus. In order to understand better the contributions of pedal mucus to a gastropod's energy budget, mucus provendering needs to be examined under a wide range of field conditions.

A serendipitous demonstration of the importance of water column characteristics on the rates of microalgal entrapment was obtained during the one-day field adhesion experiment. More microalgae were found adhering to mucous trails after one day than after seven days (Table I). This apparent contradiction of the ability of mucus to enhance microalgal biomass levels probably was due to differences in the density of phytoplankton within the water column between the two experimental periods. An algal bloom occurred during the one-day experiment (the water column was visibly green), while "normal" phytoplankton densities were present during the seven-day experiment. Mucous trails may allow limpets to use a phytoplankton bloom as an additional food resource.

Occurrence of provendering mucus

The ability of mucous secretions to stimulate the growth of potential food species is not unique to intertidal limpets. Calow (1974) documented that mucus plays a provendering role for the pulmonate *Planorbis contortus* L. The mucous trails of *P. contortus* were found to stimulate the growth of bacterial species which are ingested preferentially by *P. contortus*. The prosobranch *Hydrobia ventrosa* also is thought to use secreted mucus to trap bacteria (Kofoed, 1975a). Growth efficiency is higher in *Hydrobia* when bacteria are included in the diet (Kofoed, 1975b). Calow (1979) has outlined the circumstances when the production of a high energy provendering mucus would be adaptive. Essentially, the producer and the consumer of the mucus must be related. This can be ensured by a variety of mechanisms, including those demonstrated by *Lottia* and *C. scabra*, namely site specificity and site defense. Thus for limpets, the ability to enhance food resources seems to be related to the homing habit.

It is not known whether any other homing species use mucous trails to stimulate the growth of food species. Homing behavior depends on many factors and the homing response varies extensively even within a single species. Frequently, juvenile limpets do not home, but adults home consistently (Branch, 1981). Many gastropods and chitons have restricted microhabitats or small home ranges, and yet do not home in the strict sense of returning to precisely the same spot. The results of studies on the proportion of a limpet population showing strict homing behavior vary considerably, even when study is restricted to a single species (for the species included in this study see Hewatt, 1940; Villee and Groody, 1940; Brant, 1950; Frank, 1964; Galbraith, 1965; Jessee, 1968; Stimson, 1970; Sutherland, 1970). It is the general consensus that adult *Lottia* and *C. scabra* consistently home, but that any movement pattern for *C. digitalis* is far less regular, with movement apparently affected by both seasonal (Frank, 1964) and density-dependent factors (Breen, 1972, 1973). In spite of this variability in homing response, several gastropod and chiton species generally are considered to home. This homing response would allow the production of a high-cost, provendering mucus.

In a more general sense, mucus stimulation of bacterial and microalgal growth could be important for any organism that relies on mucus to aid in feeding. Most suspension feeders that use mucous nets are the sole consumers of their mucus; the ability of mucus to collect and stimulate the growth of entrapped food species could be exploited by the animal if the mucous traps were collected and consumed at a time that maximizes net energetic returns.

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DEFENSIVE STRATEGIES OF SOFT CORALS (COELENTERATA: OCTOCORALLIA) OF THE GREAT BARRIER REEF. II. THE RELATIONSHIP BETWEEN TOXICITY AND FEEDING DETERRENCE

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ABSTRACT

Thirty-six specimens of soft corals (Coelenterata, Alcyonacea) were tested for toxicity by exposing *Gambusia affinis* (Vertebrata, Pisces) to aqueous extracts of coral macerate and assessing mortality. Fifty percent of the soft coral extracts were determined to be ichthyotoxic to the fish, supporting earlier studies. In another experiment, commercial fish food was immersed in the same aqueous soft coral extracts, dried, and offered to *G. affinis* at three concentrations with appropriate controls. The study of feeding deterrence showed that 88% of the 36 extracts produced negative feeding responses at the highest concentration. At intermediate concentrations, 75% of the extracts acted as feeding deterrents; 48% showed detectable deterrence at lowest concentrations. Levels of toxicity and feeding deterrence, however, were not correlated; *i.e.*, feeding deterrence was as common among non-toxic corals as among toxic ones. This finding may help to explain why some soft corals, which apparently lack toxic defense substances, do not exhibit signs of predation in the field.

INTRODUCTION

One of the most important selective factors influencing the evolution of living organisms is predation. The specific morphological (*e.g.*, Kettlewell, 1956), chemical (Whittaker and Feeny, 1971), and behavioral (Harvey and Greenwood, 1978) adaptations which have evolved in plants and animals and which clearly serve an antipredatory function are extremely numerous and diverse in nature. Common antipredatory adaptations include (1) feeding deterrent properties, involving olfaction and taste, whereby an organism is avoided by a predator or receives a low food preference, and (2) toxicity, whereby an animal may ingest the prey and become ill, experience physiological stress, or die (*e.g.*, Brower, 1969). These behavioral responses in the predator can be chemically mediated by secondary compounds either produced by the prey itself (Eisner, 1970; Gerhart, 1983) or acquired by the prey in turn from its own food (Brower *et al.*, 1970; Eisner *et al.*, 1974; Schulte *et al.*, 1980; Thompson *et al.*, 1982; Carte and Faulkner, 1983; Jensen, 1984).

Evidence for co-occurrence of feeding deterrent properties and toxicity is variable. Some studies have demonstrated a positive correlation between toxicity and the presence of feeding deterrents (West, 1976; Picman *et al.*, 1982; Camazine, 1983; Camazine *et al.*, 1983; Gerhart, 1984, 1986). By contrast, Rowell *et al.* (1983) found that

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certain ferns were differentially palatable to two species of tropical grasshoppers, and that the presence of this palatability was not correlated with the presence of known plant toxins, such as phenolics and tannins. Toxicity of soft corals (Coelenterata: Alcyonacea) on the Great Barrier Reef has been demonstrated (Bakus, 1981; Coll *et al.*, 1982b; Coll and Sammarco, 1983), but the presence or absence of feeding deterrent properties has not been examined.

Soft corals can be quite common on coral reefs in the Indo-Pacific, and particularly in certain areas of the Great Barrier Reef (Dinesen, 1983). Although common species of soft coral are potentially rich sources of protein, carbohydrate, and especially lipids (Coll, 1981) and are easily accessible to predators such as fish because of their exposure, they suffer relatively low levels of predation (P.W.S., unpub. data; work in progress). Certain predators are specialized in such a way as to be able to consume highly toxic soft coral tissues. For example, *Ovula ovum* Linnaeus, 1758 (Mollusca, Prosobranchia) feeds almost exclusively on soft corals and possesses mechanisms for disposal or storage of toxins (Coll *et al.*, 1983). Certain chaetodontid fish have been observed to pick soft coral polyps from expanded colonies (Tursch and Tursch, 1982; also Sammarco, Coll, and Alino, work in progress). Most fish, however, appear to ignore the soft coral as prey; this has been attributed to their tissue toxicity (Bakus, 1981; Coll *et al.*, 1982b). In several recent studies, we have shown that approximately half of the common exposed soft corals from several regions of the Great Barrier Reef exhibit significant ichthyotoxic properties (Coll *et al.*, 1982b; Coll and Sammarco, 1983). It thus seems probable that non-toxic soft corals have some alternate or additional defensive mechanism against predation. The presence of feeding deterrents has been suggested by Tursch (1976) who pointed out that some crude soft coral extracts were highly unpalatable to fish. Stoecker (1980) also found that many tropical ascidians exhibit similar properties.

Here we report the results of a detailed study of 36 soft corals for ichthyotoxic and feeding deterrent properties as determined by tests on the mosquito fish *Gambusia affinis* (Baird and Girard) (Vertebrata, Pisces). We also examine the co-distribution of these two characters among these soft coral species and demonstrate that they do not appear to be related.

MATERIALS AND METHODS

Thirty-six common soft coral specimens (9 genera, 32 species) were collected from Britomart Reef (18°14′S, 146°45′E) (herein referred to as the BRI-series) at depths of 1–12 m, Rib Reef (18°15′S, 145°45′E) (RIB-series) at depths of approximately 5 m, and from Myrmidon Reef (18°16′S, 147°24′E) (MYR-series) at depths ranging from 2–10 m. Sampling was performed between August to October, 1982. Specimens used for toxicity and feeding deterrence studies (>20 g wet weight) were placed in labeled plastic bags and frozen. Corresponding samples were preserved in 70% ethanol and used as reference specimens for taxonomic identifications. Identifications were made using information published in Bayer (1956, 1961), Verseveldt (1980), Tixier-Durivault (1966, 1972), and references cited by these authors.

Toxicity tests

Ichthyotoxicity tests were performed according to the techniques described by Yamanouchi (1955) and Bakus and Thun (1979), using similar dosage levels. These have been described in Coll *et al.* (1982b), but a summary will be provided here.

Aqueous extracts were prepared from each soft coral as follows. Fifteen g of frozen tissue was blended with fresh water (30 ml), and the macerate were centrifuged at 10,000 RPM for 10 min. The supernatant was divided into three equal portions, two of which were used for the ichthyotoxicity study; the third was reserved for the palatability study.

The test organism used to determine the toxicity was the common mosquito fish *Gambusia affinis*. The cosmopolitan distribution of the species, its ability to survive environmental fluctuations, and its abundance make it attractive for laboratory work, especially for toxicity studies (Cornman 1968; Birkhead 1972; Spiegelstein *et al.,* 1973; Ne'eman *et al.,* 1974; Fernandez Bernaldo de Quiros, 1978). *Gambusia affinis* was chosen as the test organism because it can be trained to feed under experimental conditions in the laboratory, is discriminating in its feeding behavior, and is sensitive to feeding deterrents. These qualities have made it the preferred test organism in similar palatability tests conducted by both chemists (Rideout *et al.,* 1979) and vertebrate physiologists (Strieck, 1924; Glaser, 1966; Atema, 1977; Herbert and Atema, 1977) in studies examining the molecular basis of chemoreception in teleost fish. In addition, this extant freshwater species most likely has had no exposure to soft corals through evolutionary time and does not possess adaptations of resistance to soft coral toxins or tolerance to associated feeding deterrents.

The mosquito fish were collected from a creek at least 24 hours prior to testing and acclimatized in a large storage aquarium containing dechlorinated tap water. Only the apparently healthiest individuals of both sexes were selected for the experiment.

Extracts were added to small duplicate test aquaria, each containing fresh water (200 ml) and three randomly selected specimens of the test fish. Mortality counts were taken after 22, 45, 90, 180, 360, and 720 min for each of the extracts.

Palatability determinations

A commercial fish food in the form of dried flakes (Tetra Min^R) was used in the preparation of test foods and controls. Preliminary tests were performed to determine a concentration range for the experimental food. Concentrations were selected by determining the levels at which several randomly selected extracts could be minimally detected by the test organism. Once these levels were determined, the flakes were ground to a fine powder, from which 120 portions of 100 mg each were weighed out. Three replicate portions of fish food were prepared for each of the 36 soft coral extracts. One ml of extract was added to the first, 2 ml to the second, and 4 ml to the third. The suspensions were mixed, freeze-dried, and ground using a mortar and pestle. The food preparations were stored in the absence of moisture to prevent rehydration. Controls were prepared in a similar manner using the addition of 1, 2, and 4 ml of fresh water (instead of extract). This provided 108 test and 12 control preparations.

The method used here was particularly efficient for testing large numbers of crude extracts. Particles were clearly accepted or rejected by the fish.

Ten to twenty fish were used in the palatability tests and were conditioned to accept a small amount of untreated ground fish food from the experimenter for between 7–10 days. The test and control food preparations were presented to the fish several times per day by holding a few particles with dissecting tweezers, and releasing them just beneath the surface of the water. A small trail of dissolving chemicals was visible around each sinking particle. Responses of fish to the food were recorded only for particles detected by the fish between the surface and the mid-depth of the aquarium. Test samples and controls were presented at random, using three different samples and a control of the same concentration per feeding session.

The response of any test fish to the experimental food was classified as follows:

(1) *Acceptance:* The fish detected a food particle, seized it, and ingested it—an indication of lack of feeding deterrence;
(2) *Rejection:* The fish detected a food particle, ingested it, and expelled it immediately—an indication of feeding deterrence via taste; unpalatability;
(3) *Avoidance:* The fish detected the food and moved towards it, but turned away upon approaching it, and failed to ingest it—an indication of the presence of an olfactory deterrent.

For the purposes of this study, the term "feeding deterrence" will refer to responses including both avoidance (olfaction) and rejection (taste).

Ten unambiguous responses were recorded for each sample at a given concentration. Occasionally the fish did not detect the food (usually towards the end of an experimental feeding session). Detection could be enchanced, however, if samples and controls were presented in small quantities at different sites, decreasing the potential local build-up of food particles and their associated chemicals.

Numerical methods

Data were analyzed by non-parametric tests of association. The Kendall's Rank Correlation Coefficient (Sokal and Rohlf, 1981) yielded an indication of the level of association between toxicity and palatibility (also see Siegel, 1956).

Toxicity rankings were assigned according to relative mortality levels of test fish, using primarily the 720-min samples. Within these mortality categories, tied ranks were further split using data from successively earlier sample times. Ties unresolvable by this method were assigned according to the conventions of the Kendall's Rank Correlation Analysis (Sokal and Rohlf, 1981). Unpalatability ranks were assigned in a similar fashion, primarily using data from the most highly concentrated test preparations and ranking further on the basis of data from successively less concentrated preparations.

RESULTS

One half of the 36 soft coral aqueous extracts were found to be non-toxic; *i.e.*, they were indistinguishable from controls in their effect on *Gambusia affinis* and caused no mortality in test fish at the concentrations used (Table I). Twenty-two percent of the extracts killed all fish in 12 hours and were categorized as 100% lethal. The remainder (28%) elicited intermediate levels of mortality and were considered to be toxic. The majority of soft coral extracts examined in this study (89%, n = 36) had detectable deterrent effects on feeding in test fish at the highest concentration utilized. Seventy-five percent of the extracts elicited at least some avoidance or rejection responses by fish at the intermediate concentration, and about one-half (47%) elicited such at the lowest concentration.

The four samples (constituting 11%) showing the lowest acceptance levels were *Efflatounaria* sp. a, *Lemnalia* sp. c, *Sinularia capillosa*, and *S. flexibilis;* each showed high rejection rates at all concentrations. Two-thirds (24/32) of the samples were found to be avoided or rejected by test fish, with 50–100% rejection rates at the highest concentration. Among the least palatable of these, *Sarcophyton glaucum*, *Sarcophyton* sp. c, *Lemnalia* sp. a and b, and *Sinularia* sp. a, were rejected at the lowest concentration (at levels of ≥30%). Only eight out of 36 samples showed evidence of avoidance by the fish, including *Lemnalia* sp. a and c and *Sinularia* sp. a, which elicited avoidance responses even at the median concentration (Table I).

Two-thirds of the samples were found to be unpalatable, with high rejection scores occurring at highest concentrations. Of the 12 least palatable extracts, 5 were found to be within the "non-toxic" category, including the least palatable specimen—*Efflatounaria* sp. a (RIB-10). In addition, four of the most palatable soft coral extracts were found to fall within the lethal or toxic categories—namely *Sinularia* sp. 3 (RIB-23), *S. polydactyla*, *Sinularia* sp. c (RIB-5), and *S. peculiaris* (RIB-21). The extracts of four (4) soft corals were accepted at all concentrations; these included *Capnella* sp. a (RIB-24, -25; two specimens), *Capnella* sp. b (RIB-14), and *Sinularia* sp. c (RIB-5).

Some soft coral genera exhibited different levels of interspecific variability in palatability. For example, species of *Sinularia* were among the most variable. Various species of *Capnella* tended to elicit little feeding deterrence, while all *Lemnalia* species elicited at least some response.

There was no significant correlation between ichthyotoxicity and feeding deterrence for the 36 samples studied ($P > 0.05$, Kendall's Coefficient of Rank Correlation). Thus, no evidence was found to indicate that these two characters generally co-occur in this group of organisms.

Discussion

With respect to toxicity, the mortality data lent further support to earlier conclusions that crude aqueous extracts of some soft corals are potentially lethal to the test fish *Gambusia affinis* (see Coll *et al.*, 1982b; Coll and Sammarco, 1983). These aqueous extracts have been shown to contain suspended lipid-soluble toxins, including terpenes (Coll *et al.*, 1982b). Three very toxic samples came from alcyonacean species already found to be toxic in earlier studies: *Lemnalia* sp. c. (RIB-15), *Sinularia flexibilis* (BRI-1), and *Sarcophyton glaucum* (BRI-5) (Coll *et al.*, 1982b). The genus *Sarcophyton* was consistently found to be lethal, whereas representatives of the genus *Lemnalia* ranged from 100% lethal to non-toxic. These results are in accordance with our earlier findings.

Toxicity offers an important adaptation for organisms otherwise defenseless against predation (see Ehrlich and Raven, 1964; Eisner and Meinwald, 1966; Whittaker and Feeny, 1971; Faulkner and Giselin, 1983). Such organisms include holothurians (Bakus, 1981), sponges (Randall and Hartman, 1968; Green, 1977), crinoids (Rideout *et al.*, 1979), ascidians (Stoecker, 1980; Bakus, 1981), and the larvae of *Acanthaster planci* (crown-of-thorns starfish) (Lucas *et al.*, 1979). Saccoglossan nudibranchs are also known to exude a defensive secretion upon disturbance (Edmunds, 1966). This toxicity, although common, does not occur in the high frequency previously assumed for organisms lacking mechanisms of escape or physical defense (Bakus, 1981), such as in many of the Alcyonacea.

With respect to feeding deterrence, the soft corals examined exhibited a wide range and generally clear ranking as determined by the criteria used in this experiment. Almost 90% of all soft corals examined elicited some level of feeding deterrence in test fish. Many alcyonaceans (about 50%) on the Great Barrier Reef also exhibited ichthyotoxic characteristics. Our tests of association, however, were unable to reveal any significant relationship between these two characters. That is, the two attributes (as measured here) appear to co-occur within soft corals at random. The compounds which are responsible for the ichthyotoxic characters may well be different from those responsible for feeding deterrence. The majority of pure compounds derived from

TABLE I

Ichthyotoxicity and feeding deterrence data derived from laboratory experiments exposing Gambusia affinis *to crude soft coral extracts and food impregnated with extracts*

Species name	Specimen number	Toxicity time (Min)						Toxicity rank	Acceptance			Feeding deterrence rank
									Proportion of trails			
		22	45	90	180	360	720		1 ml/100 mg	2 ml/100 mg	4 ml/100 mg	
Category 1: 100% lethal												
Lemnalia sp. c	RIB 15	6	6	6	6	6	6	1	0.5	0.0†	0.0†	2
Sinularia capillosa	JUL 35	3	6	6	6	6	6	2	0.8	0.2	0.0	3.5
Sarcophyton glaucum	BRI 5	2	5	6	6	6	6	3	0.7	0.4	0.1	12
Sarcophyton sp. c	RIB 13	0	3	5	6	6	6	4	0.5	0.3	0.0	5
Sinularia sp. e	RIB 23	0	0	5	6	6	6	5	1.0	0.9	0.8	32
Sinularia flexibilis	BRI 1	0	0	0	2	6	6	6	0.8	0.2	0.0	3.5
Sarcophyton sp. b	JUL 4	0	0	1	2	4	6	7	1.0	0.6	0.5	22
Cladiella sp. a	RIB 12	0	0	0	0	0	6	8	1.0	0.7	0.4	9
Category 2: Toxic												
Sinularia polydactyla	JUL 1	0	0	1	1	2	4	9	1.0	1.0	0.7	30.5
Sinularia sp. c	RIB 5	0	0	0	0	2	4	10	1.0	1.0	1.0	37.5
Sinularia peculiaris	RIB 21	0	0	0	0	0	4	11	1.0	1.0	0.7	30.5
Efflatounaria sp. b	RIB 16	0	0	0	2	2	3	12	1.0	0.2	0.2†	15
Paralemnalia sp. c	RIB 9	0	0	0	0	1	3	13	1.0	1.0	0.4	20
Lemnalia sp. a	RIB 20	0	0	0	1	2	2	14	0.4	0.1†	0.1†	10
Lemnalia sp. b	RIB 8	0	0	0	1	1	2	15	0.9	0.6	0.0	8
Paralemnalia sp. b	RIB 4	0	0	0	0	1	2	16	1.0	0.9	0.4†	24
Paralemnalia sp. d	RIB 17	0	0	0	0	0	2	17	0.9	0.6	0.7	26
Lemnalia sp. d	RIB 19	0	0	0	0	0	1	18	0.9	0.3	0.5	21

Category 3: Non-toxic												
Efflatounaria sp. a	RIB 10	0	0	0	0	0	0	29.5	0.1	0.0	0.0	1
Sinularia sp. d	RIB 7	0	0	0	0	0	0	29.5	0.8	0.3	0.0	6
Sinularia sp. a	RIB 1	0	0	0	0	0	0	29.5	0.7	0.0†	0.1†	11
Sinularia mollis	BRI 6	0	0	0	0	0	0	29.5	0.8	0.6	0.0	7
Litophyton sp.	JUL 3	0	0	0	0	0	0	29.5	0.9	0.2	0.1	13
Lemnalia sp. b	RIB 6	0	0	0	0	0	0	29.5	0.7	0.1	0.3	14
Sinularia sp. g	BRI 2	0	0	0	0	0	0	29.5	0.9	0.8	0.0	9
Paralemnalia sp. a	RIB 3	0	0	0	0	0	0	29.5	0.8	0.6	0.7	16
Capnella sp. c	RIB 11	0	0	0	0	0	0	29.5	1.0	0.8	0.3	18
Dendronephthya sp.	MYR 3	0	0	0	0	0	0	29.5	1.0	0.8	0.3†	17
Sinularia sp. b	RIB 18	0	0	0	0	0	0	29.5	1.0	0.8	0.5	23
Capnella sp. d	BRI 4	0	0	0	0	0	0	29.5	1.0	1.0	0.5†	25
Sinularia variabilis	RIB 25	0	0	0	0	0	0	29.5	1.0	0.6	0.7	27
Sinularia sp. f	RIB 2	0	0	0	0	0	0	29.5	1.0	0.9	0.7	28
Sinularia dura	MYR 2	0	0	0	0	0	0	29.5	1.0	1.0	0.7†	29
Capnella sp. b	RIB 14	0	0	0	0	0	0	29.5	1.0	1.0	1.0	37.5
Capnella sp. a	RIB 24	0	0	0	0	0	0	29.5	1.0	1.0	1.0	37.5
Capnella sp. a	RIB 26	0	0	0	0	0	0	29.5	1.0	1.0	1.0	37.5
*Control one	C1	0	0	0	0	0	0	29.5	1.0	1.0	0.97	33.5
*Control two	C2	0	0	0	0	0	0	29.5	1.0	1.0	0.97	33.5
*Control three	C3	0	0	0	0	0	0	29.5	1.0	1.0	1.0	37.5
*Control four	C4	0	0	0	0	0	0	29.5	1.0	1.0	1.0	37.5

n = 10
* n = 30

Data represent number of fish (out of a total of 6) killed within time period specified (in min). Toxicity ranking also shown. Toxicity category determined by all (1), some (2), or none (3) of the fish dying. Palatability data also shown for each soft coral as indicated by acceptance by test fish of food impregnated with extract. Data represent proportion of trials (out of 10) with unequivocal acceptance at various concentrations for each extract (see text for details). † represents occurrence of avoidance response at the 10% level, except in the cases of RIB 1 and RIB 15, where the response levels were 20% and 30–40%, respectively. Feeding deterrence rank also shown. No significant association between ichthyotoxicity and feeding deterrence ($P > 0.05$, Kendall's Coefficient of Rank Correlation).

these soft corals which have been demonstrated to have ichthyotoxic properties have been terpenoids, belonging predominantly to the di- and sesquiterpene classes (Ne'eman *et al.*, 1974; Tursch *et al.*, 1978; Coll *et al.*, 1983).

Some pure sesquiterpenes have been shown to possess properties of feeding deterrence in reef fish (Tursch *et al.*, 1978), and this class of compounds is commonly found in alcyonaceans. Polyhydroxysterols also commonly occur within this group (Schmitz, 1978). These are known to be highly biologically active in certain terrestrial systems (Nakanishi, 1974), but their levels of activity, with respect to both ichthyotoxicity and feeding deterrence, specifically within the Alcyonacea, are as yet undetermined. We have shown that these classes of compounds (sterols and terpenes) are present in the aqueous extracts of soft corals (Coll *et al.*, 1982b), although they would generally be regarded as non-water-soluble compounds. The only set of toxins not assessed in our studies would be membrane-bound molecules (*i.e.*, peptide toxins) not present in the supernatant after centrifugation. Therefore, we are unable to comment on the efficacy of this latter group of compounds in the chemical defense of soft corals.

Extracts of several species of *Sinularia*, including *Sinularia* sp. a (RIB-1), *S. flexibilis* (BRI-1), *S. capillosa*, and *S. mollis* (BRI-6), were frequently rejected by the fish at low concentrations, indicating the presence of efficient feeding deterrents in the soft coral tissues. In addition, in almost all trials, *Efflatounaria* (RIB-10) was found to be clearly unpalatable. Specimens of this genus, including this one, are generally known to have little or no ichthyotoxic properties, as determined by this and previous studies. However, they are rich in terpenoid compounds (Bowden *et al.*, 1983). These compounds may be responsible for the low palatability of the tissue extracts, for such compounds are known to be unpalatable in terrestrial systems (Schwartz *et al.*, 1980).

Chemical warning signals are used by a variety of marine organisms as a defense against predation (*e.g.*, Glynn, 1980). The substances responsible for these signals are often secondary metabolites which may be toxic and can sometimes be released by the prey into its immediate surroundings. The physical basis of feeding deterrence, particularly olfaction, is generally attributed to the action of volatile substances (Kittredge *et al.*, 1974; Little, 1983). Terpenoids, although often cytotoxic, may or may not possess odorous properties. For example, the diterpene sinularin has been shown to be cytotoxic (Weinheimer *et al.*, 1977) but is certainly not volatile (pers. obs., J.C.C.). By contrast, sesquiterpenes derived from *Lemnalia* (*e.g.*, *Lemnalia* sp. c, RIB-15) are known to be ichthyotoxic (La Barre, 1984) and yet are highly volatile and pungent in odor (J.C.C. and co-workers, pers. obs.). Thus, a given compound may be an effective toxin but a poor chemical signal, as is the case in the monarch butterfly *Danaus plexippus* and its toxicity to the blue-jay *Cyanocitta cristata* (Brower, 1969). Volatility or solubility of individual terpenes within a given soft coral may be an important factor in determining the presence and intensity of their feeding deterrent capabilities.

In our study, only 22% of the soft coral extracts elicited avoidance responses, and even these were mostly at the 10% level. This indicates that olfaction was probably not a major factors in feeding deterrence. In addition, those cases where avoidance was elicited were not necessarily associated with extracts exhibiting high rejection levels. Thus, feeding deterrence via olfaction is not necessarily linked with the degree of palatability of the tissue in question. The major exception to this was the case of extracts derived from *Lemnalia* sp. c (RIB-15), where it is possible that olfaction may be at least as important in feeding deterrence as taste. This set of particularly odorous and toxic extracts deterred hungry fish from even touching the food particle on several occasions.

Common reef alcyonaceans are typically sessile, non-cryptic, and possess drab coloration. There would be a distinct adaptive advantage for such organisms to release olfactory warning substances or feeding deterrents into the water column. Since this would appear to be independent of their degree of toxicity, a type of diffuse Batesian mimicry may be occurring. Remarkably high concentrations of secondary metabolites are indeed found in some soft coral tissues, but certainly not all (Coll *et al.*, 1981; Coll and Sammarco, 1983). This is not to say that coevolution of specialized predators has not occurred. It is known that *Ovula ovum* is adapted to prey specifically on *Sarcorphyton glaucum* and detoxify the most toxic metabolite (Coll, 1983). Fish such as *Chaetodon ocellicaudus* readily feed upon such alcyonaceans as *Litophyton viridis, Sarcophyton glaucum, Clavularia inflata, Nephthea* sp., etc. (Tursch and Tursch, 1982). Other *Chaetodon* spp. are also known to feed on soft corals.

Direct extrapolation of results from laboratory data on standard test fish to field situations involving coral reef fish should be made with caution. Field observations suggest, however, that feeding deterrents are indeed effective against predation. We have observed very low levels of natural predation on these organisms in the field consistently over the past 5–6 years (pers. obs.; PWS, unpub. data).

The secondary metabolites of soft corals from the Great Barrier Reef may possess three distinct ecological functions: (1) ichthyotoxicity (Coll *et al.,* 1982b; Coll and Sammarco, 1983; this study) which may well be indicative of an anti-predatory role (Gerhart, 1984); (2) a feeding deterrent role (this study); and (3) an anti-competitor role, causing mortality in scleractinian and alcyonacean corals, demonstrated experimentally both in the laboratory and the field (Sammarco *et al.,* 1982, 1983, 1985; Coll and Sammarco, 1983; Coll *et al.,* 1982a; La Barre *et al.,* 1986). Each of these characteristics varies from species to species, both at the intergeneric and interspecific levels. The characters do not appear to be linked. Thus, feeding deterrence does not necessarily indicate toxicity, toxicity does not imply allelopathy, allelopathy does not imply feeding deterrence, and so forth. Indeed, different classes of compounds (*e.g.*, terpenes *vs.* sterols) may be responsible for these different characters in some species.

It seems highly likely that those biologically active compounds occurring within the Alcyonacea of the Great Barrier Reef, irrespective of their chemical class, serve different ecological functions, depending upon the evolutionary history of the species in question (also see Stoecker, 1980). These might include anti-predatory, anti-competitor, anti-fouling, etc. adaptations, which can occur individually or in any random combination within a species. In addition, each character can function at different levels of effectiveness in different species. Survival of individual soft corals may well be influenced by chemically mediated processes which vary in type, intensity, and co-occurrence between species. These characters have most likely played an important role in contributing to the evolutionary success of soft corals in the Indo-Pacific region.

The major findings of this study may be summarized as follows: (1) approximately 50% of the soft corals surveyed in this study from the central region of the Great Barrier Reef were found to be ichthyotoxic. These results are consistent with earlier results from other parts of the Great Barrier Reef, including the northern, central and southern regions. (2) Extracts derived from almost 90% of these same soft corals had detectable deterrent effects on the feeding activities of test fish at the highest concentrations utilized here. (3) Despite the existence of these traits, no significant association could be found between ichthyotoxicity and feeding deterrence in the soft coral species tested. That is, their co-occurrence appeared to be random. (4) It is hypothesized (a) that different chemical compounds may be responsible for the different responses in test organisms, and/or (b) that the biologically active compounds in the various species of Alcyonacea may be adapted for different functions (anti-predation, competition for space, anti-fouling, etc.) which may have evolved independently.

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CRAB PREDATION ON LIMPETS: PREDATOR BEHAVIOR AND DEFENSIVE FEATURES OF THE SHELL MORPHOLOGY OF THE PREY[1]

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ABSTRACT

The feeding behavior of rocky intertidal crabs in the tropical and temperate eastern Pacific was studied in relation to specific mechanical properties of the shells of their limpet prey. A series of laboratory experiments, involving direct observations, records of shell remains, and measurements of the forces generated by a feeding crab, showed that by far the most common feeding technique was to pry the margin of the limpet shell away from the substratum. The pattern of deformation in models of limpet shells subject to a similar prying force indicated (1) that the greatest stress on the shell was at the point of force application at the shell margin and (2) that the thickness of the shell margin contributed more to shell strength than did thickness in more apical regions of the shell. Measurements of the strength of real shells provided further support for this latter conclusion.

In addition, the strength of foot attachment, which sets the maximum prying force that the shell can experience, closely paralleled shell strength. This linkage between foot tenacity and shell strength appeared to be maintained via the degree of allometry between foot area and the thickness of the shell margin.

The potential for a particular predator feeding behavior to lead to selection for a defensive feature in shell morphology should be a function, not only of the frequency of occurrence of attacks, but also of the frequency of successful attacks. In particular, for selection to occur, some individuals must survive an attack so that they may pass on to their offspring the defensive feature that enabled survival. Compared to other crab feeding techniques, prying attacks on limpets occurred frequently and with low success. These data support the hypothesis that selection to resist prying forces has been an important feature in the evolution of limpet shell morphology.

INTRODUCTION

Selection to resist attacks by shell-breaking predators appears to have been a central feature in the evolution of the shell form of marine gastropods (Vermeij, 1977; Vermeij *et al.,* 1980, 1981). Understanding of this selection pressure requires information on both the techniques of attack used by predators and the biomechanical properties of those parts of the shell that are most important in resisting these attacks. Previous studies of this kind have focused almost exclusively on gastropods with spirally coiled shells (Kitching *et al.,* 1966; Vermeij, 1974, 1976, 1978; Zipser and Vermeij, 1978; Palmer, 1979, 1985; Bertness and Cunningham, 1981). For these species, low spires, thickened shells, narrow or occluded apertures, and strong shell sculpture

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appear to reduce the probability of mortality due to attacks by shell-breaking predators such as fish and crustaceans. Very little information is available on those morphological attributes of gastropods with patelliform (limpet-shaped) shells that help to prevent predator-induced shell breakage (but see Chapin, 1968, and Lowell, 1985, 1986, 1987).

The three major groups of shell-breaking predators that feed on limpets are crabs (Chapin, 1968; R. B. Lowell, unpub. data), fish [only certain, mostly tropical, species generate shell-breaking forces before their limpet prey are detached from the substratum (Garrity and Levings, 1983; Lowell, 1987; S. D. Gaines, pers. comm.)], and birds (Feare, 1971; Hartwick, 1976, 1978, 1981; Frank, 1982; Hockey and Branch, 1983; Mercurio *et al.,* 1985) (see also Branch, 1981, for general review). Of these three groups, crabs (or other functionally similar decapods) are perhaps the most geographically and temporally ubiquitous in the rocky intertidal areas where limpets are found in the eastern Pacific (Ricketts and Calvin, 1968; Menge and Lubchenco, 1981; Frank, 1982; Lowell, 1986).

To determine the influence these crab predators may have had on the evolution of the shell form of rocky intertidal limpets, I recorded the techniques used by crabs to feed on limpets in the tropical and temperate eastern Pacific. The results of this study indicated that by far the most frequent feeding behavior was to attempt to pry the margin of the limpet's shell away from the substratum. Therefore, I measured the contribution of the characteristically thickened margin of the shell to the breaking resistance of the shells of several eastern Pacific limpet species which commonly cooccur with these crabs. I also determined the relationship between the breaking resistance of the shell and the attachment strength of the foot.

MATERIALS AND METHODS

Crab feeding behavior

Temperate eastern Pacific. Two species of cancrid crabs, *Cancer productus* and *C. oregonensis,* were chosen to study the feeding behavior of temperate Pacific crabs. Caging experiments have shown that these predatory crabs cause significant limpet mortality in the rocky intertidal; in addition, both species will readily attack limpets in the laboratory, often within seconds of being offered limpets for the first time (R. B. Lowell, unpub. data). *Cancer productus* attains fairly large sizes; the seven individuals (6 males, 1 female) used for this study ranged from 8.2 cm to 11.5 cm in maximum carapace width. *Cancer oregonensis* is a much smaller species; the two individuals (both female) used were 3.5 cm and 5.3 cm in maximum carapace width. No molts occurred during the study. All *C. productus* and one *C. oregonensis* were collected two years prior to the study from Bamfield Inlet (48°49′N, 125°8′W) and Grappler Inlet (48°50′N, 125°7′W) near the Bamfield Marine Station, British Columbia, Canada. The other, smaller *C. oregonensis* was collected from the plankton adjacent to the marine station at the postlarval (megalops) stage two years prior to the study and raised to maturity in the laboratory. All crabs were fed only bivalves (mostly *Mytilus edulis* and *Protothaca staminea*) and barnacles (mostly *Balanus glandula*) until 23 days before the first set of observations, after which they were fed only the four sets of limpets described below. Prior to the experimental period, each crab was placed in one of nine 37.5 l glass aquaria (50 × 25 × 30 cm) with a constant supply of fresh seawater. The crabs were then left undisturbed for 23 days before being offered the first set of limpets, except for two *C. productus* which were placed in their aquaria 1 day before being offered the first limpets. These latter two crabs exhibited similar behavior to the other five *C. productus* throughout the study. To minimize disturbance, all aquaria were surrounded by black plastic sheets which admitted only dim light. The aquaria were situated in a room with a west-facing window. Although no attempt was made to regulate artificial lighting conditions exterior to the plastic enclosures, all crabs were most active during the late afternoon and through the night.

Four different size-shape classes of limpets were offered to each crab over a period of two months in 1984. To vary shape, the limpets were divided by species into a flat shell group (*Notoacmea scutum*) and a tall shell group (*Collisella pelta, N. persona*). To vary size, the limpets were further divided into small (18–23 mm in shell length) and large (30–40 mm) individuals. The four size-shape combinations were offered to the two crab species as follows: (1) five small, flat *N. scutum* per crab on 4 August; (2) four large, flat *N. scutum* per crab on 18 September; (3) five small, tall *C. pelta* per crab on 22 September; (4) four large, tall *N. persona* per crab on 24 September (*C. productus*) and 25 September (*C. oregonensis*). All crabs were offered each combination except that one *C. oregonensis* (3.5 cm) was not offered combination 4.

The limpets were collected one day before each of the observational trials from the following locations near the Bamfield Marine Station: small *N. scutum*—Kirby Point (48°51′N, 125°12′W); large *N. scutum* and *N. persona*—Ross Islets (48°52′N, 125°9′W); small *C. pelta*—Prasiola Point (48°49′N, 125°10′W). Four or five limpets were placed on each of nine flat rocks (upper surface area approximately 100 cm^2). The following day, one rock was placed in each of the nine crab aquaria during the late afternoon and, in most cases, the feeding behavior of the crabs was observed for 40–95 minutes. Following these direct observations, the limpets were left with the crabs and their fates (alive *vs.* dead, shell whole *vs.* shell broken) were recorded at 2–24 hour intervals over the next 1–5 days. Shells with minor chips that did not extend beyond the thickened part of the shell margin (such chips would not expose the soft parts of a live, attached limpet) were not recorded as broken. At the beginning of each new trial, all limpets from the previous trial were removed.

Although not studied in detail, several measurements were made of the prying forces generated by a small crab while attempting to detach a limpet. A small (5.9 cm maximum carapace width) *C. productus* was collected in Grappler Inlet and held for several months in a shallow seawater tray (approximately 70 × 70 × 20 cm) connected to the same seawater system as used for the aquaria described above. Prior to the prying force measurements, this crab was fed a combination of bivalves (mostly *P. staminea*) and limpets (mostly *N. scutum*). Although artificial lighting conditions were not controlled, this crab was also most active at night.

To measure prying force, the shell of a newly killed *N. scutum* (30.6 mm in length) was tethered by a strand of nylon filament (glued into the interior apex of the shell) running through a sheet of Plexiglas to a force transducer. The force transducer, on the opposite side of the Plexiglas sheet from the tethered shell, was positioned so as to hold the shell (via the tether) against the sheet with a force of 2.7 N. The whole setup, with the plane of the Plexiglas sheet oriented vertically, was then placed in the seawater tray at 21:30 and left until 12:30 the following day. The prying forces generated by the crab were recorded throughout this period on a strip chart recorder; the crab's behavior was also observed for the first three hours.

Tropical eastern Pacific. The feeding behavior of five species of tropical Pacific xanthid crabs was studied at the Naos Laboratory of the Smithsonian Tropical Research Institute in Panama. Two of these species (*Ozius verreuxii, Eriphia squamata*) are the most common predatory crabs co-occurring on intertidal bedrock or boulders with the tropical limpets studied (Lubchenco *et al.,* 1984). The other three (*Eurypa-*

nopeus planus, Xanthodius sternburghii, Leptodius taboganus) are more common on cobble beaches where these limpets, though present, are less common (J. H. Christy, R. B. Lowell, pers. obs.). All five species readily feed on limpets in the laboratory. Relative to *C. productus,* these crab species are all fairly small. The size ranges (maximum carapace width) and number of individuals used for each species were as follows: *O. verreuxii*—3.9–7.1 cm, 1 male, 3 females; *E. squamata*—3.0–4.5 cm, 3 males, 1 female; *E. planus*—2.1–2.3 cm, 3 males; *X. sternburghii*—2.8–3.1 cm, 10 males; *L. taboganus*—2.7 cm, 1 male. All crabs were collected from small islands (Naos, Culebra, 8°55′N, 79°32′W; Taboguilla, 8°48′N, 79°31′W) in the Bay of Panama (see Garrity and Levings, 1981, for descriptions of these islands) over several weeks prior to the observational trials.

The crabs were fed only the limpets used in these trials. For *O. verreuxii* and *E. squamata,* crabs were held individually, one in each of eight glass aquaria; individuals of each of the other three species were held together, one species in each of three glass aquaria. All aquaria (each approximately 12 l) were kept in outdoor tanks under transparent roofing where they received a constant supply of fresh seawater. No attempt was made to control artificial lighting conditions; nevertheless, the crabs were most active at night.

Three to four different size-classes (see Table V) of one limpet species (*Fissurella virescens*—tall shells) were introduced into each aquarium over a one month period (20 March–11 April 1984). Each size class was offered separately in increasing order starting with the smallest class. Although no attempt was made to standardize hunger levels, each trial was separated by at least four days. All limpets were collected from Culebra Island. The procedures for collecting the limpets and offering them to the crabs were the same as those for the temperate Pacific study.

Mechanical performance of the shell

Shell models. All limpet species used in this study from both tropical and temperate shores exhibited characteristically thickened shell margins (R. B. Lowell, unpub. data). To determine the contribution of the thickened shell margin to the strength of the shell when subject to a crab-induced prying force, I sought to compare shells with a natural thickness distribution to shells that were identical in all respects except for being of constant thickness throughout all regions of the shell. Of the several thousand eastern Pacific limpet shells that were handled during the course of this and other related studies, I never found one to have a constant thickness distribution. Therefore, it was necessary to make this comparison by constructing naturally shaped models of limpet shells. By using a homogeneous material for these models, it was also possible to avoid differences in shell strength due to differences in the thicknesses of various shell microstructures (Currey, 1980).

These homogeneous models were composed of a "fiberglass" mixture of powdered glass embedded in Coating Resin P-18 which was hardened with Catalyst P-102 (Fiberlay, Inc., Seattle, Washington). I formed the models by using a silicone rubber cast of a real *N. scutum* shell (see Fig. 3 for the dimensions and thickness profile of this shell). Four models with a natural thickness distribution were formed from full casts. Three models with a constant thickness distribution (approximately 0.8 mm thick) were formed in the following manner. A positive cast, in the form of an epoxy plug, was made from the original negative cast of the dorsal surface of the shell. By using a micromanipulator to move the positive and negative casts away

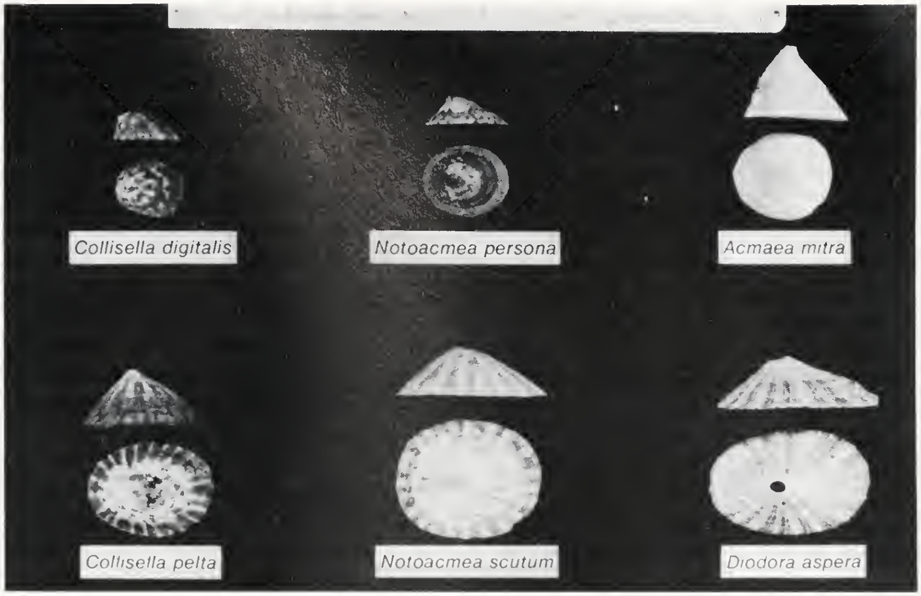

FIGURE 1. Lateral (top) and dorsal (bottom) views of temperate eastern Pacific limpet shells. Anterior side of shell faces left. 15 cm rule at top of figure.

from each other, it was possible to create an intervening space of any desired thickness which was of the same shape as the original shell. This space was filled with the "fiberglass" mixture to form the constant thickness models. The same amount of "fiberglass" was used to form both model types so that they differed only in their thickness profiles.

When a live limpet is subject to a crab-induced prying force, the force is transmitted from the shell to the foot (and, finally, to the substratum) via the horseshoe-shaped muscle scar where the foot muscle attaches to the shell. Therefore, I attached the shell models to fixed platforms with horseshoe-shaped strips of aluminum foil which were glued to the models along the ventral region of the model where this muscle scar would normally be found. A prying force, similar to that applied by a crab, was applied in a dorsal direction to the anterior margin of each model with a weighted 1.16 mm diameter steel hook of circular cross section.

To determine the patterns of deformation of the shell models, each model was coated with a spray-on brittle lacquer (Tens-Lac TL-500-75A with Undercoat U-10-A, Measurements Group, Charlotte, North Carolina). This lacquer cracks easily and the crack patterns are useful for determining the patterns of tensile strain (deformation) in a rigid structure which is subject to a force acting to deform the structure (Preuschoft *et al.,* 1975). The area where the lacquer first cracks indicates the area of greatest tensile strain. Since the shell models were made of a homogeneous material, the area of greatest strain would correspond to the area of greatest tensile stress (force/cross-sectional area). Mollusc shell material is much weaker in tension than in compression (Currey, 1980). Consequently, the area of greatest tensile stress is where the shell would most likely break.

Shell strength and foot tenacity. The strengths of various sides of the margins of real shells for several eastern Pacific limpet species also was measured. In the temper-

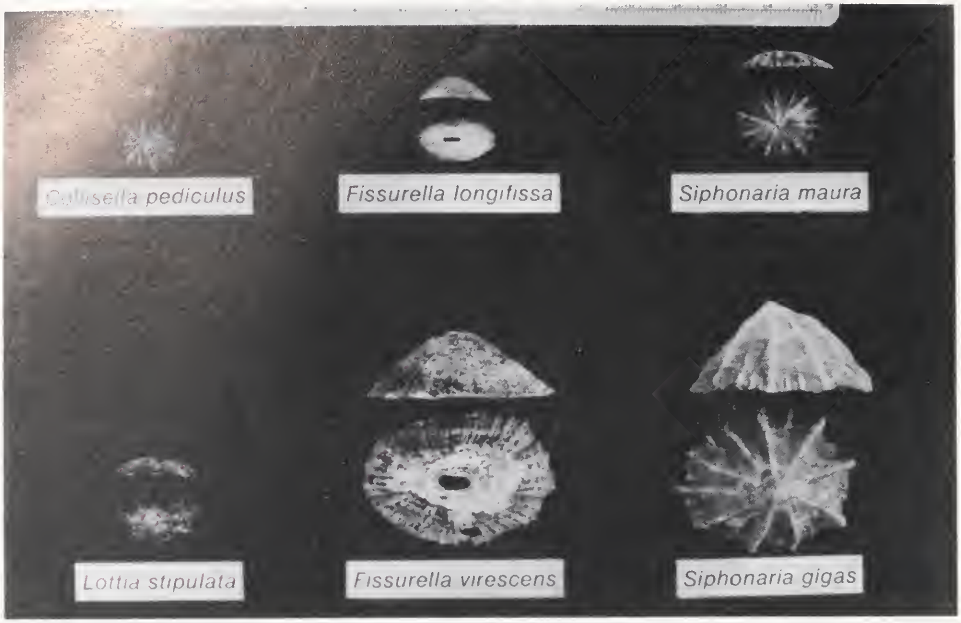

FIGURE 2. Lateral (top) and dorsal (bottom) views of tropical eastern Pacific limpet shells. See Figure 1 legend for further information.

ate eastern Pacific, measurements were taken for six common species from the west side of San Juan Island, Washington (Lime Kiln Lighthouse, 48°31′N, 123°9′W; False Bay, 48°29′N, 123°4′W): Acmaeidae—*Collisella digitalis, C. pelta, Notoacmea persona, N. scutum, Acmaea mitra;* Fissurellidae—*Diodora aspera* (Fig. 1). In the tropical eastern Pacific, measurements were taken for six additional common species from the south side of Taboguilla Island: Acmaeidae—*Collisella pediculus, Lottia* (*Scurria;* Lindberg and McLean, 1981) *stipulata;* Fissurellidae—*Fissurella longifissa, F. virescens;* Siphonariidae—*Siphonaria gigas, S. maura* (Fig. 2).

To measure the strengths of these real limpet shells, I used a procedure similar to that used for the shell models. The shells of newly killed limpets were mounted, while still wet, onto one shaft of a Monsanto (type W) tensiometer. As for the shell models, a 1.16 mm diameter steel hook (in this case, attached to the other shaft of the tensiometer) subjected the edge of the shell to a prying force. The force required to break the shell was recorded. Hooks 0.67 mm and 2.64 mm in diameter were used for particularly small and large shells, respectively (Lowell, 1987); hook diameter had no significant effect on the force needed to break the shells (Lowell, 1985).

To mount the shells securely enough to withstand the force required to break the shell, it was necessary to provide a greater surface area for the glue to attach than the area of the muscle scar. Since the shell model experiment showed that the area of greatest stress in a shell subject to a prying force is at the point of force application at the margin of the shell (see Results), the real shells were mounted via two steel cables inserted into a pool of quick-setting epoxy put into the interior apical region of the shell dorsal to and overlapping the edges of the muscle scar. This method of mounting was double-checked by mounting lacquer-coated shell models (3 natural thickness, 4 constant thickness) in the same manner and subjecting them to prying forces. This

TABLE I

Regressions of foot length (FL, in mm) against shell length (SL, in mm) for eastern Pacific limpet species

Species	Equation	Range	n	r	*P*
TEMPERATE					
Acmaea mitra	FL = −2.13 + 0.755SL	10.5–33.5	12	0.9888	<0.0001
Collisella digitalis	FL = −1.61 + 0.743SL	11.8–25.2	10	0.9889	<0.0001
Collisella pelta	FL = 0.38 + 0.689SL	13.1–33.9	10	0.9865	<0.0001
Notoacmea persona	FL = −2.55 + 0.753SL	10.0–31.8	11	0.9958	<0.0001
Notoacmea scutum	FL = 1.07 + 0.612SL	23.1–49.7	11	0.9892	<0.0001
Diodora aspera	FL = 1.98 + 0.757SL	21.2–52.5	11	0.9648	<0.0001
TROPICAL					
Collisella pediculus	FL = 0.38 + 0.551SL	6.0–15.0	9	0.9318	0.0003
Lottia stipulata	FL = −2.13 + 0.761SL	13.3–21.1	10	0.9617	<0.0001
Fissurella longifissa	FL = 0.67 + 0.662SL	10.2–19.2	10	0.9808	<0.0001
Fissurella virescens	FL = −0.85 + 0.716SL	13.9–40.4	10	0.9863	<0.0001
Siphonaria gigas	FL = −2.33 + 0.748SL	8.7–48.1	12	0.9904	<0.0001
Siphonaria maura	FL = −1.80 + 0.905SL	10.4–18.7	10	0.9742	<0.0001

Range indicates maximum and minimum values for shell lengths used in regressions. n—sample size; r—correlation coefficient; *P*—probability that r = 0.

mounting procedure yielded the same results as those reported for the more natural procedure of mounting the models with strips of aluminum foil attached only to the region of the model where the muscle scar would normally be. The quick-setting epoxy generated heat while setting; to minimize any potential effects of this heating on the strength of the real shells, the dorsal surface of each shell was kept immersed in seawater while the epoxy was setting. All shells broke at the shell margin rather than around the apical region.

The maximum possible prying force that the margin of a limpet shell can experience is set by the maximum strength of attachment of the foot to the substratum (maximum tenacity). Therefore, one would expect selection for the strength of the shell margin to be sensitive to maximum tenacity (Lowell, 1985, 1987). I used spring scales to measure the maximum tenacity of previously undisturbed, healthy limpets on flat rock surfaces (except for two *S. gigas,* see below) in the field. These measurements were made by subjecting the margin of the shell to a prying force in a manner identical to and on the same sides of the shell as for the shell strength measurements. In addition, the limpets used in the tenacity measurements came from the same populations as those used in the shell strength measurements. On average, the Panamanian limpets attain much higher tenacities than the northeastern Pacific limpets (Lowell, 1987) and, possibly in consequence, the incidence of damage to the foot during detachment was much greater for the Panamanian limpets.

Both shell strength and foot tenacity were measured for limpets of a variety of different sizes. Foot area, estimated from measurements of shell length and width, was chosen as a measure of size because of its relevance to tenacity. Tenacity was always measured after the foot had tightly clamped to the substratum. Therefore, maximum foot length (FL) and width (FW) were measured for several individuals (collected from the same populations used for the shell strength and foot tenacity measurements) of each species while they were tightly clamped to transparent glass plates (FL for both *Siphonaria* species included the length of the ventral surface of the head, which was also used for adhesion by these two species). The relationship of shell length and width to foot length and width was then calculated (Tables I, II). Foot

TABLE II

Regression of foot width (FW, in mm) against shell width (SW, in mm) for eastern Pacific limpet species

Species	Equation	Range	n	r	*P*
TEMPERATE					
Acmaea mitra	$FW = -1.30 + 0.795SW$	8.7–28.8	12	0.9729	<0.0001
Collisella digitalis	$FW = -1.33 + 0.750SW$	8.5–20.1	10	0.9613	<0.0001
Collisella pelta	$FW = 1.58 + 0.675SW$	9.1–27.0	10	0.9827	<0.0001
Notoacmea persona	$FW = -1.63 + 0.729SW$	7.7–26.6	11	0.9878	<0.0001
Notoacmea scutum	$FW = -1.29 + 0.761SW$	17.8–39.5	11	0.9768	<0.0001
Diodora aspera	$FW = -1.11 + 0.957SW$	14.4–34.4	11	0.9530	<0.0001
TROPICAL					
Collisella pediculus	$FW = -0.17 + 0.627SW$	5.1–11.2	9	0.9455	0.0001
Lottia stipulata	$FW = -0.99 + 0.795SW$	9.5–15.6	10	0.9395	0.0001
Fissurella longifissa	$FW = 0.94 + 0.641SW$	6.5–11.1	10	0.9720	<0.0001
Fissurella virescens	$FW = 0.99 + 0.622SW$	9.1–29.1	10	0.9847	<0.0001
Siphonaria gigas	$FW = -1.25 + 0.700SW$	6.1–40.9	12	0.9976	<0.0001
Siphonaria maura	$FW = 0.21 + 0.640SW$	7.1–14.5	10	0.9556	<0.0001

Range indicates maximum and minimum values for shell widths used in regressions.

area (FA) was calculated as the area of an ellipse: $FA = 0.25\ \pi FL \cdot FW$ (Miller, 1974; Dimock, 1984).

One Panamanian species, *S. gigas,* is usually found on the backs of conspecifics when small. Therefore, the tenacities of the two smallest individuals used for this species were measured while on the backs of larger individuals. The two smallest *S. gigas* used for the shell strength measurements also came from the backs of larger individuals. Both the shell strengths and foot tenacities of these small *S. gigas* fell on the regression lines calculated for the larger *S. gigas.* Consequently, all sizes were pooled for the regressions given in Tables VI and VII.

Statistics. The limpets used in the following regression analyses were chosen so as to provide a fairly even distribution of sizes within the size ranges tested. In some cases, the data were log-transformed before analysis to linearize the data and homogenize the variances. For those analyses where neither the X nor Y variable could be regarded as the independent variable and where the regression equation was to be used for functional (slopes and intercepts to be compared among regressions) rather than predictive (regressions used merely to predict Y for a given X) purposes, the reduced major axis was calculated rather than the standard least squares regression line (Ricker, 1973, 1984).

RESULTS

Crab feeding behavior

Direct observations. The large temperate Pacific species, *C. productus,* exhibited four techniques for feeding on the limpets they were offered (Table III). (1) Pry—During this behavior, the tip of a chela or walking leg was inserted under the edge of a shell and an attempt was made to pry the shell away from the substratum. In most cases the tip of the appendage was not fully under the edge of the shell and the attempt was unsuccessful. None of the crab species showed an obvious preference for a particular side of the shell. Rather, they usually probed around the edge of the shell until they found a sufficiently large space under the shell margin to initiate a prying

TABLE III

Direct observations of feeding techniques used by crabs

					Feeding Technique											
					Prying			Lateral slide			Crush at apex			Crush at margins		
Crab species	Number of crabs	Limpet species	Limpet size (mm)	Dur obs	U	S	%	U	S	%	U	S	%	U	S	%
Cancer productus	7	*Notoacmea scutum* f	18–23	40	38	0	0	0	0	—	0	0	—	0	0	—
Cancer productus	7	*Notoacmea scutum* f	30–40	95	82	0	0	0	0	—	0	0	—	0	0	—
Cancer productus	7	*Collisella pelta* t	18–23	60	15	9	38	0	1	100	0	0	—	0	7	100
Cancer productus	7	*Notoacmea persona* t	30–40	90	284	8	3	0	0	—	3	1	25	0	0	—
				totals	419	17	4	0	1	100	3	1	25	0	7	100
Cancer oregonensis	2	*Notoacmea scutum* f	18–23	40	12	0	0	0	0	—	0	0	—	0	0	—
Cancer oregonensis	2	*Notoacmea scutum* f	30–40	95	28	0	0	0	0	—	0	0	—	0	0	—
Cancer oregonensis	2	*Collisella pelta* t	18–23	50	0	0	—	0	0	—	0	0	—	0	0	—
				totals	40	0	0	0	0	—	0	0	—	0	0	—

Dur obs—duration of observations in minutes; U—number of unsuccessful attacks observed; S—number of successful attacks observed; %—percent successful attacks observed; f—flat, t—tall.

attack. All successful prying attacks which were directly observed resulted in the shell being detached whole. (2) Lateral slide—In one case, a *C. productus* successfully used its chela to slide a small, high-spired *C. pelta* off the rock in a lateral direction. (3) Crush at apex—Four attempts were made to crush the apex of large, tall *N. persona* between the two fingers of the chela. The apex of this species, though elevated, is rounded and provides a poor grip for an attacking crab; only one attempt was successful. (4) Crush at margins—Seven attempts by *C. productus* were made to crush the shells of small, steep-sided *C. pelta* between the fingers of one chela where each finger was placed at opposite margins of the shell. This technique was always immediately successful. The prying technique was by far the most common feeding behavior observed for *C. productus* (prying *vs.* all other techniques, $\chi^2 = 441$, df = 1, $P < 0.0001$).

This latter tendency was even more pronounced for the smaller crab species. The prying technique was the only behavior observed for *C. oregonensis* (Table III). The Panamanian crabs were more reluctant to feed while being observed and direct observations of their feeding behavior were not quantified. Nevertheless, of more than fifty observed attacks by *O. verreuxii* and *E. squamata*, only the prying technique was seen.

Shell remains. The high frequency of prying attacks also may be inferred from the high frequency of whole shells that were found during 1–8 days of feeding (Tables IV, V). The only other observed feeding behavior which resulted in shells being removed whole, the lateral sliding technique, was very rare (Table III). Since all crab species frequently broke up limpet shells after they were removed, most of the % whole values less than 100 in Tables IV and V probably greatly underestimate the frequency of successful prying attacks relative to other types of attacks. The shells of all or most of the limpets killed by the smallest crabs (*C. oregonensis, E. planus, X. sternburghii, L. taboganus*) were removed whole. The relative frequency of whole *versus* broken shells increased with increasing limpet size for the other crab species, although one comparison was not significant: *C. productus*—flat limpets (*N. scutum*), shell condition (whole or broken) *vs.* size, $\chi^2 = 6.75$, df = 1, $P = 0.0094$; *C. produc-*

TABLE IV

Condition (whole vs. *broken) of shells of limpets offered to temperate crabs*

Crab species	Number of crabs	Limpet species	Limpet size (mm)	Dur obs	Whole	Broken	Alive	% Whole
Cancer productus	7	*Notoacmea scutum* f	18–23	2	0	11	24	0
Cancer productus	7	*Notoacmea scutum* f	30–40	3	8	10	10	44
Cancer productus	7	*Collisella pelta* t	18–23	1	13	22	0	37
Cancer productus	7	*Notoacmea persona* t	30–40	1	14	10	4	58
Cancer oregonensis	2	*Notoacmea scutum* f	18–23	2	4	1	5	80
Cancer oregonensis	2	*Notoacmea scutum* f	30–40	3	6	1	1	86
Cancer oregonensis	2	*Collisella pelta* t	18–23	4	10	0	0	100
Cancer oregonensis	1	*Notoacmea persona* t	30–40	5	4	0	0	100

Dur obs—Duration of observations in days; Whole—number of shells known to be detached whole; Broken—number of shells broken either during or after detachment; Alive—number of limpets alive at end of feeding trial; % Whole—percent known to be detached whole of those eaten; f—flat, t—tall.

tus—tall limpets (*C. pelta, N. persona*), shell condition *vs.* size, $\chi^2 = 2.58$, df = 1, $P = 0.109$; *O. verreuxii*—*F. virescens*, % whole *vs.* size (Spearman's Rank Correlation), r = 0.8531, n = 12, $P = 0.0047$; *E. squamata*—*F. virescens*, % whole *vs.* size (Spearman's Rank Correlation), r = 0.7937, n = 12, $P = 0.0085$. Most of the increased frequency of whole shells observed for the larger limpets was probably due to decreased breakage while manipulating the shell after being removed. Some of this increase in whole shell frequency, however, was probably also due to an increase in prying attacks as the ratio of limpet size to crab size increased.

TABLE V

Condition (whole vs. *broken) of shells of* Fissurella virescens *offered to tropical crabs*

Crab species	Number of crabs	Limpet size (mm)	Dur obs	Whole	Broken	Alive	% Whole
Ozius verreuxii	4	15–20	1	3	14	3	18
Ozius verreuxxi	4	25–30	1	13	5	2	72
Ozius verreuxii	3	35–40	1	11	0	2	100
Ozius verreuxii	1	40–45	1	3	0	0	100
Eriphia squamata	4	15–20	1	1	17	2	6
Eriphia squamata	4	25–30	1	9	3	8	75
Eriphia squamata	4	35–40	1	5	1	10	83
Eurypanopeus planus	3	15–20	6	3	0	2	100
Eurypanopeus planus	3	20–30	6	3	0	2	100
Eurypanopeus planus	3	35–40	1	1	0	3	100
Xanthodius sternburghii	10	15–20	6	2	0	3	100
Xanthodius sternburghii	10	20–30	6	3	0	2	100
Xanthodius sternburghii	10	35–40	1	2	0	2	100
Leptodius taboganus	1	15–20	8	1	0	4	100
Leptodius taboganus	1	20–30	8	1	0	3	100
Leptodius taboganus	1	35–40	4	1	0	3	100

See Table IV legend for further information.

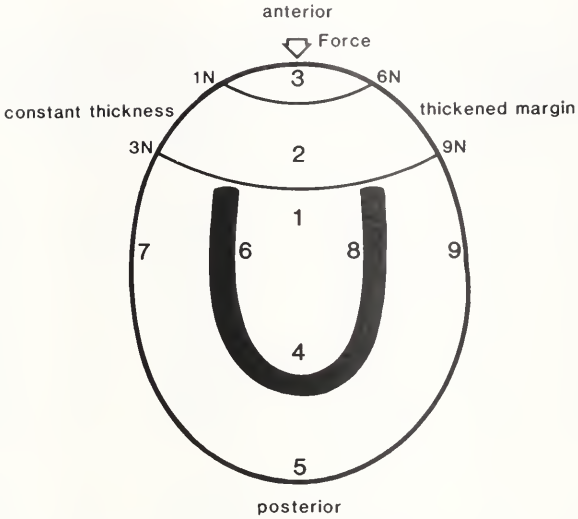

FIGURE 3. Diagrammatic ventral view of interior of shell model. The thin semicircular lines show the extent of deformation (as indicated by cracks in the brittle lacquer) at two levels of loading at the anterior edge of the model (arrow indicates where force applied; direction of force is into plane of figure). The values on the left are the forces that were required to deform the constant thickness models to the indicated radial distances from the point of force application. Those on the right are the forces required to deform the thickened margin models a similar amount. The shaded, horseshoe-shaped region shows approximately where the foot muscle would attach to the shell. Numerals 1–9 indicate the locations where shell thickness was measured on the *Notoacmea scutum* shell used to make the models. Location 1 was the apex. Locations 2, 4, 6, and 8 were in the thin annular region surrounding the apex. Locations 3, 5, 7, and 9 were at the thickest part of the shell margin. Thicknesses: 1—0.67 mm, 2—0.35 mm, 3—1.23 mm, 4—0.50 mm, 5—1.10 mm, 6—0.57 mm, 7—1.31 mm, 8—0.53 mm, 9—1.38 mm. Dimensions of whole shell: length—38.7 mm, width—31.7 mm, height—11.5 mm.

Prying force. Since no other food was available, the small *C. productus* used for the prying force measurements spent most of the night (22:30–09:30) attempting to pry the tethered shell away from the Plexiglas sheet. During this period, 610 separate prying forces were recorded. Most were 1–5 s in duration, although a few lasted up to 20 s. The peak force recorded was 10.1 N. The prying forces were generated with the tips of the walking legs or chelae and all sides of the shell were attacked. This left a record of tiny chips around the entire margin of the shell, although the thickened part of the margin remained intact.

Mechanical performance of the shell

Shell models. As the prying force was increased, the lacquer coating of all shell models first cracked on the ventral side of the shell at the point of force application. As the force was further increased, semicircular cracks formed farther from this point, creating the same concentric pattern on all models (Fig. 3). This indicates that the greatest stress was at the point of force application. In a detailed study of scallop shells subject to point forces, Pennington and Currey (1984) also measured a tendency for shell deformation to be greater near the point of force application.

Figure 3 also shows, for the two thickness distributions, the approximate force required to form cracks out to the indicated radial distances from the point of force application. The absolute magnitudes of these forces are unimportant since they are

TABLE VI

Equations for shell strength (F, in newtons) as a function of foot area (FA, in cm²) for all species and sides of shell

Species	Side	Equation	Range	n	r	*P*
TEMPERATE						
Acmaea mitra	A	$F = 54.4FA^{0.58}$	0.34–2.50	11	0.8131	0.0023
Collisella digitalis	A	$F = 42.3FA^{0.83}$	0.28–1.80	12	0.7769	0.0030
Collisella digitalis	P	$F = 33.2FA^{1.00}$	0.29–1.78	8	0.9231	0.0011
Collisella pelta	A	$F = 11.8FA^{1.31}$	1.18–4.35	11	0.7948	0.0035
Collisella pelta	R	$F = 13.6FA^{1.13}$	0.73–4.22	8	0.9709	0.0001
Collisella pelta	P	$F = 10.1FA^{1.28}$	0.80–3.34	7	0.9501	0.0010
Notoacmea persona	A	$F = 27.5FA^{0.63}$	0.19–2.95	8	0.8452	0.0012
Notoacmea scutum	A	$F = 11.6FA^{0.94}$	0.76–8.04	20	0.9091	<0.0001
Notoacmea scutum	R	$F = 13.0FA^{0.98}$	1.10–4.30	8	0.9077	0.0018
Notoacmea scutum	P	$F = 9.0FA^{1.10}$	1.19–5.91	8	0.9652	0.0001
Diodora aspera	A	$F = 14.0FA^{0.71}$	1.80–8.23	8	0.9180	0.0013
TROPICAL						
Collisella pediculus	A	$F = 76.5FA^{0.82}$	0.20–0.74	11	0.8306	0.0015
Lottia stipulata	A	$F = 28.6FA^{0.79}$	0.41–1.49	12	0.7654	0.0037
Fissurella longifissa	A	$F = 45.3FA^{1.53}$	0.37–1.65	12	0.9560	<0.0001
Fissurella virescens	A	$F = 40.1FA^{1.16}$	0.25–4.13	12	0.9815	<0.0001
Fissurella virescens	R	$F = 58.4FA^{0.91}$	0.34–6.37	12	0.9546	<0.0001
Fissurella virescens	P	$F = 47.0FA^{1.25}$	0.21–4.19	12	0.9813	<0.0001
Siphonaria gigas	A	$F = 62.0FA^{0.90}$	0.10–6.77	11	0.9873	<0.0001
Siphonaria maura	A	$F = 36.3FA^{1.10}$	0.53–1.58	9	0.7845	0.0123

Equations detransformed from linear regressions of ln F *vs.* ln FA (r and *P* values given for ln-ln regressions). Range indicates maximum and minimum values for foot areas used in regressions. Side: A—anterior, R—right, P—posterior.

specific to the artificial material used for the models. Of significance is the result that much greater forces were required to cause a given degree of deformation or stress for the natural, thickened margin models as compared to the constant thickness models. Since the total amount of material used in each model type was equal, the constant thickness models had thicker apical regions and thinner shell margins than the natural thickness models. Therefore, these results indicate that, with respect to the strength of the shell when resisting prying forces, marginal thickness is of more importance than is thickness in more apical regions of the shell.

Shell strength and foot tenacity. For each species and side of the shell tested, the natural logarithm (ln) of the force required to break the shell (shell strength) and ln of the force required to detach the foot (foot tenacity) were regressed against ln foot area. In all cases, shell strength and foot tenacity showed a highly significant increase with increasing size (Tables VI, VII).

DISCUSSION

Crab feeding behavior

Three major patterns were evident in the results of the feeding experiments. First, the prying technique was clearly the most frequently observed feeding behavior for all crabs, including the seven *C. productus* and one *C. oregonensis* which had not fed on limpets for at least two years. Furthermore, the other *C. oregonensis*, which had been collected from the plankton, had never fed on limpets and was only observed

TABLE VII

Equations for foot tenacity (F, in newtons) as a function of foot area (FA, in cm²) for all species and sides of shell

Species	Side	Equation	Range	n	r	*P*
TEMPERATE						
Acmaea mitra	A	$F = 26.4FA^{0.77}$	0.25–4.29	10	0.9742	<0.0001
Collisella digitalis	A	$F = 21.9FA^{0.89}$	0.25–2.97	9	0.9782	<0.0001
Collisella digitalis	P	$F = 23.3FA^{0.85}$	0.32–1.52	8	0.8839	0.0036
Collisella pelta	A	$F = 7.6FA^{1.29}$	0.69–3.89	20	0.9278	<0.0001
Collisella pelta	R	$F = 8.8FA^{1.50}$	0.82–3.71	8	0.9600	0.0002
Collisella pelta	P	$F = 9.6FA^{1.57}$	0.91–3.36	8	0.9902	<0.0001
Notoacmea persona	A	$F = 21.1FA^{0.96}$	0.59–3.26	8	0.9945	<0.0001
Notoacmea scutum	A	$F = 8.1FA^{1.08}$	0.32–5.93	23	0.9662	<0.0001
Notoacmea scutum	R	$F = 12.9FA^{0.90}$	0.64–5.98	10	0.9215	0.0002
Notoacmea scutum	P	$F = 12.6FA^{0.92}$	0.90–5.46	8	0.9524	0.0003
Diodora aspera	A	$F = 7.4FA^{0.97}$	1.45–9.20	12	0.8841	0.0001
TROPICAL						
Collisella pediculus	A	$F = 27.8FA^{0.94}$	0.17–0.73	13	0.7673	0.0022
Lottia stipulata	A	$F = 26.0FA^{0.86}$	0.36–2.06	12	0.9652	<0.0001
Fissurella longifissa	A	$F = 41.4FA^{1.38}$	0.48–1.01	8	0.8913	0.0030
Fissurella virescens	A	$F = 33.1FA^{1.05}$	0.49–4.23	14	0.9571	<0.0001
Fissurella virescens	R	$F = 31.8FA^{1.07}$	0.39–3.26	9	0.9669	<0.0001
Fissurella virescens	P	$F = 33.4FA^{0.93}$	0.62–3.37	11	0.9806	<0.0001
Siphonaria gigas	A	$F = 32.9FA^{1.03}$	0.09–4.87	11	0.9834	<0.0001
Siphonaria maura	A	$F = 25.7FA^{1.06}$	0.42–2.00	10	0.9208	0.0002

See Table VI legend for further information.

to use the prying technique. These results indicate that the initial preference for prying attacks is not learned. Moreover, this pattern was maintained through two months of being fed only limpets, indicating that learning does not greatly change the strong preference for prying attacks (see Hughes, 1980, Lawton and Hughes, 1985, and references therein for discussions of the role of learning in the feeding behavior of crabs).

A second pattern observed was the increase in the relative frequency of prying attacks as the ratio of limpet size to crab size increased. When the ratio of limpet size to crab size is large, some of the other possible techniques (*e.g.*, crush at margins) become physically impossible. Other techniques become very difficult (*e.g.*, apex crush). In contrast, even small crabs can pry off large limpets if the limpet is caught before it has clamped down. This also should be true for lateral sliding attacks and the reason for the low frequency of this behavior is unknown. This low frequency may be related to the tendency for the edge of the shell to dig into the substratum when it is slid sideways. Undoubtedly, the prying forces generated by crabs include a lateral, in addition to a vertical, component. A switch in feeding behavior as the ratio of gastropod size to crab size increases has also been observed for crabs feeding on spirally coiled marine gastropods (Bertness and Cunningham, 1981; Reimchen, 1982; ap Rheinallt and Hughes, 1985; Lawton and Hughes, 1985).

A third pattern emerging from these observations was an increase in the relative frequency of prying attacks for flat shells as opposed to tall shells. This was probably due to the difficulty of attaining a purchase on flat shells for non-prying attacks.

In addition to differences in the frequency of occurrence of attacks, the various feeding techniques also differed in the frequency of success (Table III). The potential

TABLE VIII

Correlation matrix among thicknesses at several locations (1–7) on the shells of Notoacmea scutum

	1	2	3	4	5	6	7
1	1.0000						
2	0.8043	1.0000					
3	0.4973	0.7444	1.0000				
4	0.7716	0.8838	0.5636	1.0000			
5	0.5288	0.7973	0.9642	0.6373	1.0000		
6	0.7397	0.9072	0.6867	0.9081	0.7562	1.0000	
7	0.5582	0.7938	0.9599	0.6595	0.9680	0.7568	1.0000

n = 20 for all correlations. See Figure 3 for positions of locations.

for selection for defensive features of shell morphology is a function of both of these factors (Vermeij, 1985). For example, this point was illustrated in the one case where the crabs were large enough and the limpets small enough and of the right shape to enable the marginal crushing technique (*C. productus*—*C. pelta*). Combining all the attack techniques used in this case, over half the observed attacks were immediately successful (17 successful *vs.* 15 unsuccessful; Table III) and all limpets were eaten within four hours. For selection to favor a particular defensive feature of morphology, individuals possessing that feature must possess a higher probability of successfully surviving an attack than those lacking that feature. If the probability of surviving a series of attacks is vanishingly small regardless of morphology (as seems to be the case for marginal crushing attacks), selection is unlikely to occur (see Reimchen, 1980; Vermeij, 1982, 1985; Sih, 1985, for further discussion). The much higher probability of surviving a prying attack (Table III), coupled with the high frequency of this behavior, suggests that selection for morphological features of the shell which enhance resistance to prying forces is quite strong.

Mechanical performance of the shell

The results from the crab-behavior and the limpet-shell-model experiments emphasize the importance of the marginal thickness of limpet shells as a morphological

TABLE IX

Equations for shell strength (F, in newtons) as a function of shell thickness (T, in mm) at seven locations on the shells of Notoacmea scutum

Location	Equation	Range	r	*P*
1	$F = 57.3T^{1.00}$	0.23–1.50	0.6581	0.0016
2	$F = 160.3T^{1.45}$	0.15–0.66	0.7704	0.0001
3	$F = 44.6T^{1.39}$	0.31–1.57	0.8966	<0.0001
4	$F = 83.5T^{1.18}$	0.20–1.04	0.7030	0.0005
5	$F = 35.4T^{1.38}$	0.33–1.94	0.9054	<0.0001
6	$F = 92.1T^{1.26}$	0.20–1.07	0.7460	0.0002
7	$F = 35.2T^{1.53}$	0.39–1.72	0.9260	<0.0001

See Figure 3 for positions of locations. Equations detransformed from linear regressions of ln F *vs.* ln T (r and *P* values given for ln-ln regressions). Range indicates maximum and minimum values for shell thicknesses used in regressions. n = 20 for all equations.

TABLE X

Equations for thickness at margin of shell (T, in mm) as a function of foot area (FA, in cm^2) for all species and sides of shell

Species	Side	Equation	Range	n	r	*P*
TEMPERATE						
Acmaea mitra	A	$T = 1.03FA^{0.43}$	0.34–3.84	16	0.9221	<0.0001
Collisella digitalis	A	$T = 0.67FA^{0.63}$	0.28–1.80	24	0.8153	<0.0001
Collisella digitalis	P	$T = 1.04FA^{0.56}$	0.28–1.80	24	0.8939	<0.0001
Collisella pelta	A	$T = 0.37FA^{0.78*}$	0.69–4.55	88	0.8263	<0.0001
Collisella pelta	R	$T = 0.45FA^{0.73*}$	0.69–4.55	88	0.8765	<0.0001
Collisella pelta	P	$T = 0.44FA^{0.75*}$	0.69–4.55	88	0.8875	<0.0001
Notoacmea persona	A	$T = 0.57FA^{0.36}$	0.19–2.95	8	0.8940	0.0027
Notoacmea scutum	A	$T = 0.40FA^{0.66*}$	0.59–8.04	106	0.9180	<0.0001
Notoacmea scutum	R	$T = 0.48FA^{0.63*}$	0.59–8.04	106	0.9239	<0.0001
Notoacmea scutum	P	$T = 0.46FA^{0.68*}$	0.59–8.04	106	0.9234	<0.0001
Diodora aspera	A	$T = 0.51FA^{0.65*}$	1.80–8.23	8	0.9752	<0.0001
TROPICAL						
Collisella pediculus	A	$T = 1.47FA^{0.59}$	0.16–0.80	19	0.8702	<0.0001
Lottia stipulata	A	$T = 0.79FA^{0.49}$	0.41–1.49	15	0.7491	0.0013
Fissurella longifissa	A	$T = 0.94FA^{0.70*}$	0.30–1.65	13	0.9763	<0.0001
Fissurella virescens	A	$T = 0.93FA^{0.52}$	0.21–6.37	43	0.9604	<0.0001
Fissurella virescens	R	$T = 1.12FA^{0.55*}$	0.21–6.37	43	0.9702	<0.0001
Fissurella virescens	P	$T = 0.99FA^{0.58*}$	0.21–6.37	43	0.9613	<0.0001
Siphonaria gigas	A	$T = 1.29FA^{0.43*}$	0.10–7.26	16	0.9720	<0.0001
Siphonaria maura	A	$T = 0.85FA^{0.79*}$	0.31–1.58	16	0.7296	0.0013

Equations detransformed from linear regressions (reduced major axis) of ln T *vs.* ln FA (r and P values given for ln-ln regressions). Range indicates maximum and minimum values for foot areas used in regressions. Side: A—anterior, R—right, P—posterior. *—indicates significant ($P < 0.05$) allometric increase or decrease of marginal thickness with increasing foot area, as indicated by an exponent that is significantly greater or less than 0.5. See Clarke, 1980, for significance tests for slopes of reduced major axis regressions.

defense against crab predation. This conclusion is further supported by the results of the strength measurements for real shells. For the strength measurements of the anterior side of *N. scutum,* thickness measurements at several locations on the shell were taken before the shells were broken. These locations corresponded to locations 1–7 of Figure 3 and included three positions along the shell margin and four positions in the apical region. Due to the high correlations between these thickness measurements, particularly between the measurements of marginal thickness (Table VIII), it was not feasible to analyze the relationship between shell strength and thickness using a single multiple regression (Bendel, 1971). Therefore, shell strength was instead regressed separately against each measure of shell thickness. The correlation coefficients for these regressions indicate that marginal thickness accounted for more of the variation in the strength of these real shells than did any of the apical thicknesses (Table IX).

The importance of marginal thickness to the strength of real limpet shells was further emphasized by comparisons among all the species tested. As for shell strength, marginal thickness also showed a highly significant increase with increasing size for all species and sides of the shell measured (Table X). These thickness measurements corresponded to the same sides of the shell for which shell strength and foot tenacity were measured. The measurements were made on the individuals used for the shell strength measurements (before breaking) and were supplemented with measure-

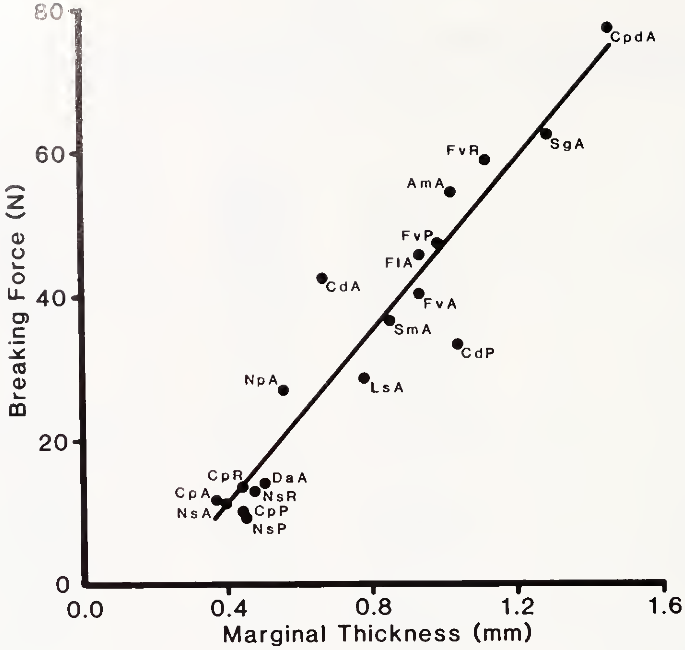

FIGURE 4. Force to break shell (F, in newtons) *versus* marginal thickness (T, in mm) at intermediate size (foot area = 1 cm^2) for all species and sides of shell. Values calculated from equations in Tables VI and X. The regression line indicates the significant increase of shell strength with increasing marginal thickness: $F = -12.6 + 59.1T$; $n = 19$; $r = 0.9517$, $P < 0.0001$. Temperate limpets: Am, *Acmaea mitra;* Cd, *Collisella digitalis;* Cp, *C. pelta;* Da, *Diodora aspera;* Np, *Notoacmea persona;* Ns, *N. scutum.* Tropical limpets: Cpd, *Collisella pediculus;* Fl, *Fissurella longifissa;* Fv, *F. virescens;* Ls, *Lottia stipulata;* Sg, *Siphonaria gigas;* Sm, *S. maura.* Sides of shell: A, anterior; R, right; P, posterior.

ments made on additional individuals from the same populations. For those species with radial ribs extending to the shell margin, thickness was calculated as the average of the rib and adjoining furrow thicknesses.

These ln-ln regressions (Tables VI, X) were then used to calculate shell strength and marginal thickness for each species and side of the shell at a single intermediate size (foot area = 1 cm^2) common to all species. These two sets of measurements were regressed against each other and shell strength showed a highly significant increase with increasing marginal thickness (Fig. 4).

The prying forces exerted by crab predators on limpet shells differ from the breaking forces exerted by crabs on the shells of spirally coiled gastropods in that the maximum possible prying force on a limpet shell is set by foot tenacity (Lowell, 1985, 1987). This intrinsic limit does not apply to spirally coiled gastropods, the shells of which still provide protection from predators even after the foot is detached. Therefore, limpets are unique in that one would expect selection to act to link the mechanical performances of the shell and the foot, given a cost to excessively strengthening the shell (Palmer, 1981). This linkage has been demonstrated in interspecific comparisons of limpets in the eastern Pacific (Lowell, 1987).

Further, intraspecific evidence for the linkage of the performances of the shell and foot is evident in comparisons of the slope of ln shell strength regressed against ln foot area to the slope of ln foot tenacity regressed against ln foot area for each species

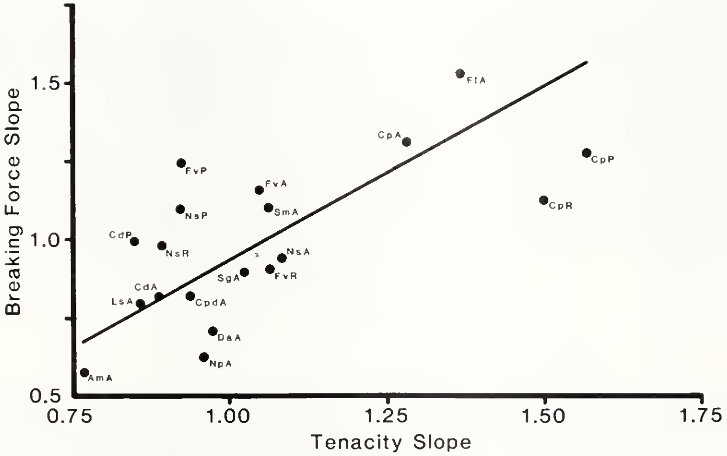

FIGURE 5. Slopes of shell strength ln-ln regressions (SS) versus slopes of foot tenacity ln-ln regressions (FT) for all species and sides of shell. Slopes given as exponents in Tables VI and VII. The regression line (reduced major axis) indicates a significant tendency for greater slopes for shell strength to be associated with greater slopes for foot tenacity: SS = −0.18 + 1.11FT; n = 19; r = 0.6708; $P = 0.0017$. See Figure 4 for abbreviations.

and side of the shell tested (slopes given in detransformed form as exponents; Tables VI, VII). These two slopes differed significantly in only one of nineteen cases (*F. virescens*—posterior side; Lowell, 1987). This means that, for the most part, the shell strength and foot tenacity ln-ln regressions were essentially parallel. Thus, the ratio of shell strength to foot tenacity remained fairly constant with increasing size. This linkage between shell strength and foot tenacity over a wide range of different slopes is further emphasized by the highly significant correlation between the slopes for shell strength and those for foot tenacity for all species and sides of the shell combined (Fig. 5).

The linkage between shell strength and foot tenacity appears to be at least partially due to the degree of allometric increase of marginal thickness with increasing size. For an isometrically growing limpet, marginal thickness should increase as the square root of foot area due to simple geometric considerations. Several exponents in Table X were significantly different than 0.5, indicating an allometric change in marginal thickness with increasing foot area. Furthermore, the exponents in Table X (indicating degree of allometry) were highly correlated with the exponents for shell strength as a function of foot area in Table VI (Fig. 6). In other words, the rate of increase of shell strength with increasing size appears to be linked to the rate of increase of marginal thickness with increasing size. This suggests that the limpets can control shell strength so that it parallels foot tenacity by controlling the degree of allometry in marginal thickness.

Taken as a whole, these data underscore the relationship between the localized forces generated by crabs feeding on limpets and the localized thickening (= strengthening) of a specific region of the limpet shell, the shell margin. The strengths of whole shells have also been reported for a few species of bivalves (Elner, 1978; Currey, 1979; Blundon and Kennedy, 1982; Boulding, 1984) and spirally coiled gastropods (Currey, 1979; Vermeij and Currey, 1980; Currey and Hughes, 1982; Blundon and Vermeij, 1983). These measurements were all made by crushing whole shells between

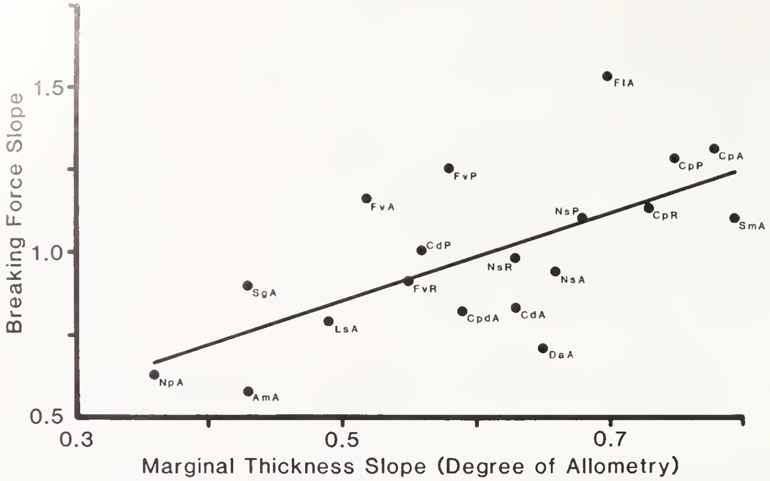

FIGURE 6. Slopes of shell strength ln-ln regressions (SS) versus slopes of marginal thickness ln-ln regressions (MT) for all species and sides of shell. Slopes given as exponents in Tables VI and X. SS represents the rate of increase of shell strength with increasing foot area. MT represents the rate of increase of marginal thickness with increasing foot area (= degree of allometry). SS and MT are positively correlated: SS = 0.19 + 1.33MT; n = 19; r = 0.6589; $P = 0.0022$. See Figure 4 for abbreviations.

planar or rounded surfaces. In most cases, the force was applied to opposite sides of whole shells (left and right valves still joined for bivalves) in various orientations across all or much of the entire width of the shell. This kind of "whole-animal" crushing force is generated by certain species of fish (Palmer, 1979) and crabs [when mollusc size/crab size is small (bivalves—Elner, 1978; Blundon and Kennedy, 1982; Boulding, 1984) (gastropods—Zipser and Vermeij, 1978; Bertness, 1981; Bertness and Cunningham, 1981; Reimchen, 1982; ap Rheinallt and Hughes, 1985; Lawton and Hughes, 1985)].

In contrast, the above studies on crabs have shown that, when the ratio of mollusc size to crab size is large, crabs exhibit a strong tendency to attack the edge of the valve for bivalves or the shell lip, apex, or similar narrow region of the shell for spirally coiled gastropods. These studies have also shown that the probability of an unsuccessful attack is much greater when the ratio of mollusc size to crab size is large. As discussed earlier, this suggests that with respect to crab predation, the potential for selection for the strength of these localized regions of the shell may be greater than for the strength of other regions of the shell. Therefore, measurements of the force required to crush whole shells across the region of greatest width should be used with caution in discussions of the evolution of defensive shell morphologies. In those cases, however, where the thicknesses of different regions of the shell are correlated (as was found for *N. scutum;* Table VIII), such "whole-animal" strengths may be correlated with the strengths of the more critical regions of the shell.

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SEASONAL ALLOCATION OF RESOURCES TO GROWTH OF SHELL, SOMA, AND GONADS IN *MERCENARIA MERCENARIA*

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ABSTRACT

Thirteen monthly measurements of individually marked juvenile (16 mm long) and adult (60 mm long) specimens of *Mercenaria mercenaria* (L.) from field plots in North Carolina demonstrated similar seasonal patterns in size-adjusted monthly growth rates in shell volume: a large absolute maximum occurred in spring (April or May) with smaller relative maxima in mid summer and late autumn. The ratio of juvenile to adult size-adjusted growth rates in shell volume was nearly constant for ten months but then increased eight-fold in December and January. This growth anomaly between size classes could not be explained by examining dry weights of soma and gonads from additional marked juvenile and adult *Mercenaria* that were sacrificed monthly. Juveniles differed from adults by possessing negligible gonadal mass on all dates. However, knowledge of monthly changes in adult gonadal mass did not explain a significant amount of the residual variation in the regression of monthly juvenile volumetric growth on monthly adult volumetric growth. Seasonal changes in growth of adult gonadal mass and quarterly examinations of gonad histology both suggested a winter period of negligible gametogenesis followed by a spring burst of intense reproductive activity. The best explanations for the anomalously high volumetric growth of juveniles relative to adults in December and January are: (1) winter availability of a food source accessible only to juveniles or (2) biochemical storage of energy during winter by adults in preparation for the process of rapid gametogenesis in spring. If the latter explanation is correct, adult *Mercenaria* exhibit a large seasonal change in the allocation of resources between somatic growth and reproduction with maximal allocation to reproduction in winter months before gonad histology and growth of gonadal mass indicate reproductive effort.

INTRODUCTION

A complete understanding of the life history of any organism requires knowledge of how its resources are apportioned between potentially competing demands and whether that allocation of resources changes over time with either age or season (Williams, 1966; Calow, 1979). Life-history theory has usually focused on the consequences of various strategies of allocating resources between growth and reproductive effort (Stearns, 1976; Caswell, 1982). Most multicellular animals and higher plants undergo an initial period of growth and development before attainment of sexual maturity and production of gametes. Consequently, an ontogenetic shift in the relative allocation of available resources away from somatic growth and towards reproductive output is widespread (see Caswell, 1982; Bayne and Newell, 1983).

In this paper, we provide results from an empirical examination of how the bi-

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valve mollusc *Mercenaria mercenaria* allocates resources to various processes (growth in internal shell volume, somatic mass, and gonadal mass) during each month of a complete year. We compare two size classes, reproductive adults and juveniles of a size that exhibits negligible gonadal development, as a means of inferring how attainment of sexual maturity alters seasonal patterns of growth in *Mercenaria.* Surprisingly, we show that adult growth in shell volume is a relatively constant multiple of juvenile growth for 10 months of the year until December and January, when it declines by about 85%. These were not the two coldest months of the year. We argue that this unexpected seasonal divergence in growth rates of the two size classes is best explained by either the winter availability of a food source unique to the smaller clams or a winter shift in resource allocation by the adults into biochemical energy and nutrient storage in preparation for the massive reproductive activity of early spring.

MATERIALS AND METHODS

To estimate the monthly volumetric growth of juvenile (pre-reproductive) and adult (reproductive) *Mercenaria mercenaria* (L.) in a North Carolina estuary, we placed 36 juveniles and 12 adults (all individually marked and measured) into each of 10 1-m^2 field enclosures on 19 December 1981. We then excavated these marked clams from the enclosures on about the 19th (15th to 20th) of each of the following 12 months and remeasured and returned them to their assigned enclosures. Measurements were made to the nearest 0.1 mm by vernier calipers in each of three perpendicular linear dimensions (length, height, thickness). Internal shell volume (V) was calculated on each date for each individual clam from those three measurements (in cm) using the regression $V = 1.64 + 0.22\ L \times H \times T$ ($r^2 = 0.997$) from Peterson (1983). Enclosures used to confine these clams consisted of 1-m^2 square fences of 6-mm mesh, anchored by metal stakes at the corners and at midpoints of each side. The mesh was 15 cm high but forced $\simeq$10 cm vertically into the sediments so that only $\simeq$5 cm projected above the sediment surface. Such enclosures allowed the clams to burrow into and live in natural sediments on the bottom. Clams were individually marked with redundant pairs of color-coded dots of Mark-Tex Corp. paints on the outer surface of each valve.

The field site used for these manipulations was a protected embayment in Middle Marsh within Back Sound, North Carolina, near Cape Lookout. This site has been described previously (marked as M II in Fig. 1 of Peterson *et al.,* 1983). It was on a shallow bottom that was almost always covered by water and was exposed to air during only $\simeq$20 occasions annually. Sediments were muddy sands with almost equal % by weight of sands (53%) and muds (47%). Nearby measurements of water temperature (Sutherland and Karlson, 1977) suggest an annual range of monthly means from 4–29°C, although greater extremes are probably encountered in shallow areas like those where our enclosures were located. Because of the nearby (3 km) Beaufort Inlet, salinities remain high year-round (above 32‰ except after heavy rainstorms: unpub. data from H. J. Porter, University of North Carolina, for similar and adjoining Bogue Sound).

To estimate the monthly changes in biomass of gonads and soma for both the juvenile and adult clams, we erected 24 additional 1-m^2 enclosures into which were placed 12 adult and 36 juvenile *Mercenaria.* Juveniles were defined by size for this entire set of manipulations (these 24 and the other 10 enclosures) with lengths varying only slightly (mean lengths ± SD ranging across enclosures from 13.8 ± 1.6 to 16.0

± 1.0). We also kept the adult size ranges low and identical across all 34 enclosures with mean lengths ± SD varying from 59.0 ± 4.8 to 61.9 ± 4.9. On the same 12 dates when clams were remeasured in the 10 enclosures used to estimate individual growth rates, all clams from two of the additional 24 enclosures were collected, returned to the laboratory, and frozen at −10°C. In addition, two sets of identically handled and selected clams were frozen on the 19 December 1981 starting date to provide estimates of initial biomass values. Only after all 13 collections had been completed and held frozen for 6–18 months were clams individually thawed, measured in all three linear dimensions, and dissected into separate, pre-weighed Al weighing dishes for gametes and soma. A single individual performed all dissections in a random order within a three-month period so as to minimize variability in the somewhat subjective methodology of separating gametes from soma. These two separate components were then dried at 60°C to constant (<0.1% variation) weight (3–8 days) and weighed to the nearest 0.001 g. Clams in this group used for sacrificial mass analysis were not marked and followed as individuals, but were marked with a single paint color to identify them as members of the experimental group.

To test whether the seasonal pattern of gamete development in gonads of adult *Mercenaria* during our 12-month study corresponded to the published descriptions (Porter, 1964) of reproductive development in *Mercenaria* in this same area of North Carolina, we selected three adult and five juvenile *Mercenaria* for histological analysis on each of four sampling dates (March, June, September, and December 1982). These individuals were haphazardly chosen from among those collected for estimation of gonadal and somatic mass, with at least one individual selected from each enclosure. These clams were opened alive in the laboratory on the day of collection and the soft tissues removed and fixed in Bouin's solution. After 24–33 months the fixative was removed with 70% ethanol and tissues were infiltrated with paraffin, sectioned at 5 μm, and stained with Harris-modified hematoxylin (without acetic acid) and eosin Y in alcohol. We then described the condition, appearance, and abundance of gametes on each slide (which included at least one adult male and one adult female at each sampling date) under a Wild M20 microscope and compared them to the seasonal descriptions of *Mercenaria* gonad histology in Loosanoff (1937a,b), Keck *et al.* (1975), and Eversole *et al.* (1980).

All 34 enclosures used to confine clams for our subsequent measurements were initially cleared of all *Mercenaria* and other large benthic macroinvertebrates by first using fingers to plow systematically the entire 1-m^2 surface area and then twice sieving *in situ* the top 5–8 cm through 3-mm mesh. We performed this initial clearing so as to help homogenize the local environments for each of our sets of clams. This desire to maintain similarity among replicate enclosures also led to our use of identical initial densities of both juveniles and adults across all 34 enclosures. During all 12 subsequent samplings (achieved with the same methodology), we continued to remove all macroinvertebrates other than our marked *Mercenaria*. The *Mercenaria* used in our manipulations were all treated similarly; for the 60 days prior to being placed into field enclosures, they were held in the laboratory in a running seawater system.

RESULTS

Average density of the initially marked *Mercenaria* specimens inside the field enclosures gradually declined over the 12-month observation period (Table I). Declines in the average density of clams in the adult size class (≃60 mm in length) were small

TABLE I

Average densities (±SD) of both juvenile and adult Mercenaria mercenaria *recovered alive each month per 1-m² enclosure for: (1) those 10 1-m² enclosures resampled each month to estimate individual growth and (2) the two enclosures destructively sampled each month for mass analyses*

	Growth enclosures (n = 10)		Mass enclosures (n = 2)[b]	
Sampling month[a]	Juveniles	Adults	Juveniles	Adults
January	30.0 (2.3)	12.0 (0)	21.0 (1.4)	11.5 (0.7)
February	29.2 (2.5)	12.0 (0)	22.0 (2.8)	11.5 (0.7)
March	27.4 (2.1)	12.0 (0)	21.0 (1.4)	11.5 (0.7)
April	25.8 (2.5)	12.0 (0)	27.0 (0)	12.0 (0)
May	23.1 (3.7)	12.0 (0)	26.0 (0)	10.0 (0)
June	15.9 (3.0)	11.9 (0.3)	16.0 (2.8)	11.0 (1.4)
July	10.9 (5.1)	11.7 (0.5)	21.0 (7.1)	11.5 (0.7)
August	10.2 (5.2)	11.4 (0.7)	21.0 (5.7)	11.5 (0.7)
September	9.1 (5.8)	11.3 (0.8)	12.0 (5.7)	11.5 (0.7)
October	8.7 (5.8)	11.2 (1.0)	16.0 (5.7)	12.0 (0)
November	7.5 (4.9)	11.2 (1.0)	6.5 (0.7)	10.0 (2.8)
December	7.2 (4.9)	11.1 (1.0)	13.5 (2.1)	11.5 (0.7)

[a] Initial densities of both size classes of *Mercenaria* were set equal in December (31 days before the January sampling) at 36 m^{-2} for juveniles (13–16 mm long) and 12 m^{-2} for adults (59–62 mm long). Sampling occurred on about 19th of each month.

[b] Average densities do not exhibit an uninterrupted decline over time in the mass enclosures because two enclosures were sacrificially sampled (out of an initial 24) each month, whereas the same 10 growth enclosures were resampled every month.

(<10%) in both types of enclosure: (1) those resampled each month to estimate individual growth rates in shell size and (2) those destructively sampled after varying time periods to estimate the tissue mass of individual clams in each size class. In contrast, recovery of living, marked juveniles declined greatly over the 12 months: by ≃80% in the growth enclosures and 60% in the mass enclosures. Despite the reductions in numbers of marked clams, we began with sufficiently large numbers that all average densities greatly exceeded zero, even in December, and thus permitted our intended analyses of monthly growth rates.

Because growth rates of *Mercenaria* and other invertebrates slow as the animals grow, we first had to remove from our raw growth data the influence of changing body size before we could estimate the unbiased monthly variation in growth rate. This was especially true for the juvenile size class, which increased in average internal shell volume by a factor of three over 12 months (from 525 to 1869 mm^3), but it also applied to the adult size class, whose surviving members increased by about 20% in average internal shell volume (from 37,538 to 43,372 mm^3). To remove the effect of changing body size from observed data on change in internal shell volume, we performed for each size class (juvenile and adult) a separate regression of observed volumetric change against initial shell volume for each month, using as individual data points all surviving clams recovered at the end of that month, independent of enclosure (Table II). We then calculated from each regression the size-adjusted mean growth in internal shell volume (±1 SE) for a clam of fixed initial size (19 mm in length ≃1097 mm^3 in volume for the juvenile group and 60 mm in length ≃37,538 mm^3 in volume for the adult group).

These size-adjusted monthly growth increments in internal shell volume (Fig. 1)

TABLE II

Regression equations[a] and fits for successive monthly relationships between shell volume at the start of a month (x) and change in shell volume over the month (y)

	Adults				Juveniles			
Month[b]	a	b	n	r^2	a	b	n	r^2
Jan	2205	−0.058	118	0.06[c]	−23.1	0.10	290	0.15[c]
Feb	753	−0.009	115	0.00	−15.2	0.12	246	0.17[c]
Mar	2274	−0.038	111	0.08[c]	3.9	0.20	239	0.45[c]
Apr	2466	−0.030	115	0.03[c]	34.7	0.30	236	0.46[c]
May	2130	−0.019	118	0.01	−13.5	0.20	214	0.35[c]
Jun	2020	−0.041	119	0.06[c]	−11.2	0.05	133	0.12[c]
Jul	26	0.010	117	0.01	−13.5	0.12	78	0.08[c]
Aug	1456	−0.022	114	0.02	−154	0.15	77	0.32[c]
Sep	863	−0.021	110	0.02	−6.8	0.03	63	0.06[c]
Oct	−11	0.003	108	0.00	29.0	0.02	64	0.02
Nov	300	0.004	109	0.00	117	0.06	60	0.11[c]
Dec	531	−0.013	109	0.01	114	0.03	56	0.09[c]

[a] Parameters in linear regression $y = a + bx$; n is sample size, r^2 is proportion of variance explained by regression. Volume units are mm^3.

[b] For each month, growth equation applies to the time period that started on the 19th of the previous month and ended on the 19th of the month listed.

[c] $P < 0.05$ (all r^2-values without marks are non-significant: $P > 0.05$).

demonstrate a clear seasonality of growth rates for both size classes of *Mercenaria* in Back Sound, North Carolina. Standard errors are small, showing that the temporal variability patterns are real. Both juveniles and adults exhibited similar seasonal variation in size-adjusted volumetric growth with three relative maxima: a large absolute maximum in spring (April–May), and smaller ones in mid summer (July or August) and late autumn (November). Late summer (August–October for juveniles and September–October for adults) was a period of low size-adjusted volumetric growth, while adults also exhibited a second low-growth period in early winter (December–January). By calculating the ratio of size-adjusted mean juvenile growth rate to size-adjusted mean adult growth rate for each month of the year (Fig. 2), it is clear that adults grew at a relatively constant multiple of juvenile growth for 10 months of the year. Then in December and January, the ratio of juvenile to adult growth increased by about 8-fold. This ratio of juvenile to adult growth for December and January falls significantly above the 99% C.I. based on the mean ratio and its variance for the other 10 months.

Because juvenile mortality was sufficiently high to reduce greatly the numbers of clams contributing to monthly estimates of volumetric growth toward the end of the 12-month period (Table I), it was conceivable that our estimates of juvenile growth may have been biased in later months if mortality was in any way dependent upon growth rate. To test this possibility, we compared the monthly mean volumetric growth of only those juveniles that were recovered and measured every month (18 individuals) to the monthly mean volumetric growth of juveniles as estimated in Figure 1 from all individuals that survived any given month. These two estimates of monthly growth of juveniles of size 19 mm were highly correlated ($r^2 = 0.86$) and the regression coefficient (slope) between them was 1.00. This analysis suggests that our volumetric growth estimates based upon the maximum possible number of surviving

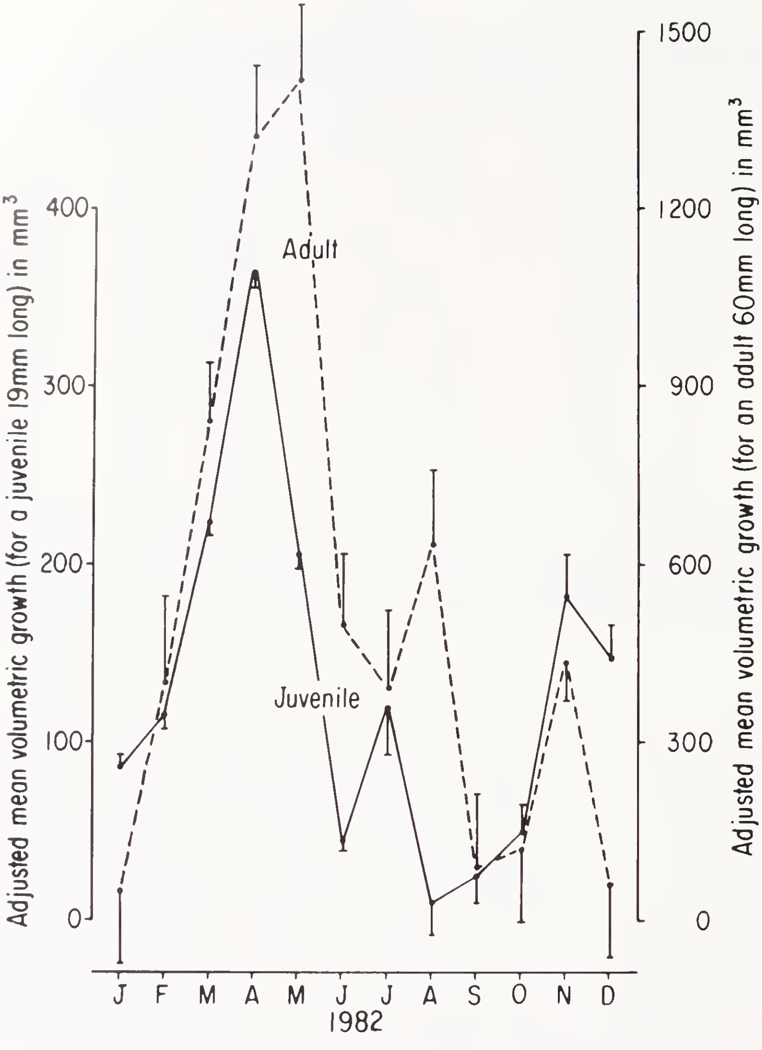

FIGURE 1. Mean volumetric growth (±SE) each month, adjusted to a constant size of clam: 19 mm long for juveniles and 60 mm long for adults. Adjustments to constant size were made from monthly regressions for each size class (juvenile and adult) of volumetric growth against initial shell volume for all surviving individuals in that month. Scales are adjusted to permit an approximate superimposition of the two plots. Sample sizes for each mean can be obtained from Table I.

juvenile *Mercenaria* (Fig. 1) were not biased by mortality selecting according to growth rate over the 12-month period.

To estimate mean monthly growth in mass adjusted for a clam of constant initial size, we first regressed mass against volume for each of the 13 months, calculating a separate linear regression equation for juvenile and adult size classes. These regressions (Table III) used all individual clams pooled from both of the replicate enclosures. For each month, we then used (1) the observed mass-volume relationship for

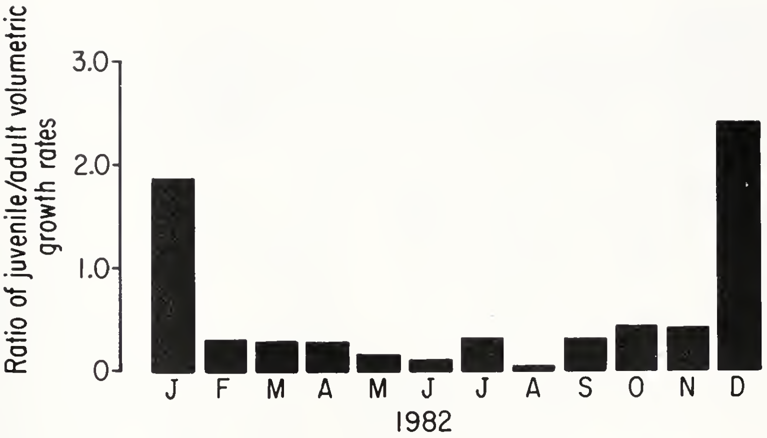

FIGURE 2. Monthly variation in the ratio of adjusted volumetric growth rates of juveniles to adults. Data come from Figure 1.

the beginning of the month to estimate the average biomass at the start of that month of a clam of fixed initial size (1087 mm^3 for juveniles and 37,538 mm^3 for adults) and (2) the observed mass-volume relationship at the end of the month to estimate the average biomass of a clam that had grown to the new size specified by the curve (Fig. 1) of adjusted monthly volumetric growth. By subtracting the initial mass from the final mass, we estimated the average monthly growth in biomass adjusted to a clam of fixed initial size. This process was done separately for mass of soma and gametes

TABLE III

Regression equations[a] *and fits for successive monthly relationships between internal shell volume (x) of* Mercenaria *and* (1) *somatic mass or* (2) *gonadal mass (y) for juvenile and adult size classes*

	Adult soma				Adult gonads				Juvenile soma			
Month	a	b	n	r^2	a	b	n	r^2	a	b	n	r^2
Dec	0.61	3.3×10^{-5}	20	0.30[b]	0.03	2.2×10^{-6}	20	0.06	−0.004	6.0×10^{-5}	20	0.85[b]
Jan	−0.40	5.6×10^{-5}	23	0.82[b]	−0.19	8.0×10^{-6}	23	0.51[b]	0.021	2.3×10^{-5}	41	0.03
Feb	0.80	2.9×10^{-5}	23	0.13	−0.14	7.3×10^{-6}	23	0.26[b]	0.020	3.3×10^{-5}	44	0.20[b]
Mar	0.49	3.3×10^{-5}	22	0.78[b]	−0.12	7.4×10^{-6}	22	0.28[b]	0.008	3.7×10^{-5}	43	0.85[b]
Apr	−0.36	5.8×10^{-5}	23	0.86[b]	−0.42	1.8×10^{-5}	23	0.52[b]	0.001	4.8×10^{-5}	53	0.88[b]
May	0.03	4.6×10^{-5}	20	0.84[b]	−0.27	1.3×10^{-5}	20	0.53[b]	0.006	4.2×10^{-5}	44	0.91[b]
Jun	0.71	2.7×10^{-5}	22	0.49[b]	0.11	4.5×10^{-7}	22	0.00	0.009	3.4×10^{-5}	27	0.82[b]
Jul	−0.59	5.7×10^{-5}	23	0.77[b]	−0.15	4.7×10^{-6}	23	0.42[b]	0.003	4.0×10^{-5}	42	0.87[b]
Aug	0.56	3.2×10^{-5}	22	0.49[b]	−0.01	3.1×10^{-6}	22	0.16	0.006	4.2×10^{-5}	42	0.90[b]
Sep	−0.13	4.6×10^{-5}	23	0.93[b]	−0.14	6.7×10^{-6}	23	0.54[b]	−0.064	7.9×10^{-5}	18	0.41[b]
Oct	0.63	2.7×10^{-5}	27	0.28[b]	0.06	9.7×10^{-7}	27	0.02	0.018	3.2×10^{-5}	32	0.46[b]
Nov	−0.04	4.1×10^{-5}	20	0.88[b]	−0.08	2.5×10^{-6}	20	0.25[b]	0.023	3.1×10^{-5}	13	0.91[b]
Dec	0.10	3.6×10^{-5}	20	0.55[b]	0.05	1.5×10^{-7}	20	0.00	0.040	3.0×10^{-5}	23	0.50[b]

[a] Parameters in linear regression $y = a + bx$; n is sample size; r^2 is proportion of variance explained by regression. Juveniles possessed negligible gonadal mass in all months. Volumes are in mm^3; masses are in g.

[b] $P < 0.05$ (all r^2-values without marks are non-significant: $P > 0.05$).

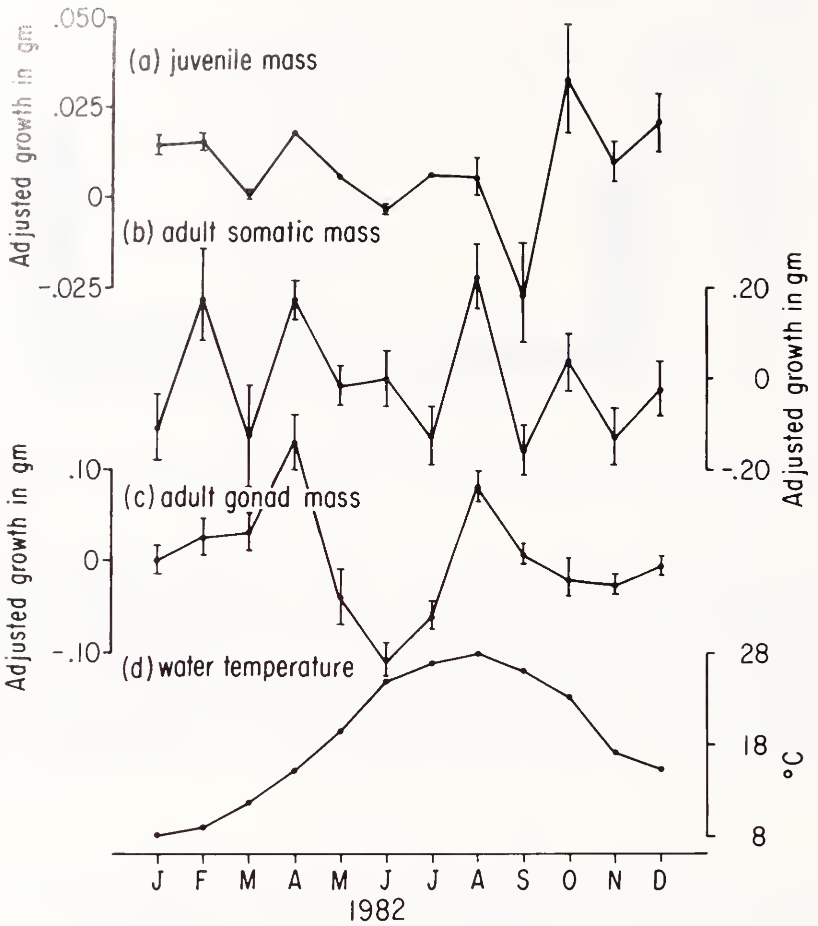

FIGURE 3. Monthly changes in average adjusted growth (a) in total biomass (all of which was soma) for juveniles of 19 mm size, (b) in somatic mass, and (c) in gonadal mass for adults of size 60 mm. Curve (d) is the mean daily water temperature (averaging daily maxima and minima) for each month from continuous records taken by W. W. Kirby-Smith at Duke University Marine Laboratory at nearby Taylor's Creek. Note that each monthly datum refers to data for the preceding month (for example, the January data refer to change from 19 December to 19 January). Bars indicate ±SE (n ranging from 38–86). Some error bars are smaller than the symbols.

for both juveniles and adults. This procedure is analogous to that used by Hilbish (1986) for similar purposes. Results are plotted in Figure 3, along with a curve of average daily water temperature for each month taken at a nearby (4 km) location in Back Sound. The monthly temperature datum plotted is the arithmetic mean value of the daily averages of observed maximum and minimum for all days that were included in that particular time interval (28–32, depending upon how close to the 19th each sampling occurred).

The gonadal mass in the juveniles was negligible (<0.01% of total mass) on every sampling date, so that only the curve of size-adjusted growth in somatic mass is plotted for this size class in Figure 3. Size-adjusted growth rates of gonadal mass for adults

TABLE IV

Correlation coefficients (r) calculated over 12 months between pairs of variables: arithmetic mean of daily water temperatures for each day of the month (Temp.), adjusted mean growth of internal shell volume (Vol. growth) for juveniles (19 mm-long) and adults (60 mm-long), adjusted mean growth of biomass (Mass growth) of soma for juveniles and adults and of gonads for adults[a]

		Vol. growth		Mass growth		
Variables	Temp.	Juv.	Adult	Juv.	Adult soma	Adult gonads
Temp.	1.00	−0.49	−0.05	−0.37	0.00	−0.29
Vol. growth-Juv.		1.00	+0.70[b]	+0.28	+0.08	+0.43
Vol. growth-Adult			1.00	0.00	+0.33	+0.32
Mass growth-Juv. soma				1.00	+0.40	+0.16
Mass growth-Adult soma					1.00	+0.56
Mass growth-Adult gonads						1.00

[a] Actual data given in Figures 1 and 3.

[b] $P < 0.05$ (all without marks are non-significant: $P > 0.05$).

exhibited a seasonal pattern of positive growth in late winter (February–March) with a peak in April, followed by a summer period of substantial negative growth (May–June–July). Size-adjusted growth of gonadal mass for adults was then positive again in August and virtually static (near zero) for September through January. Size-adjusted growth rates in somatic mass varied greatly from month to successive month in adults, and did not exhibit any smooth seasonal changes for either adults or juveniles. The direction of change between successive months in size-adjusted growth of somatic mass generally corresponded for the two (adult and juvenile) curves: in 8 of 11 cases the two curves moved in the same direction.

Correlation coefficients (Table IV) calculated between all possible pairs of variables (arithmetic mean water temperature, juvenile size-adjusted volumetric growth, adult size-adjusted volumetric growth, juvenile biomass growth, adult somatic mass growth, and adult gonadal mass growth) revealed only one statistically significant ($\alpha = 0.05$) relationship: a positive one (r = .70) between size-adjusted volumetric growth of juveniles and adults. Water temperature was not significantly correlated with any of the growth variables. The relationship between size-adjusted somatic growth rates of juveniles and adults was positive (r = .41) but non-significant. Similarly, size-adjusted adult somatic growth and gonadal growth were positively related (r = .56) but the relationship was not statistically significant ($\alpha = 0.05$). Analogous multiple regression analyses, done to explain observed monthly variation in size-adjusted volumetric growth rates of adult *Mercenaria,* demonstrated that addition of monthly variation in size-adjusted growth of gonadal mass in adults did not explain any of the unexplained variance around the regression of size-adjusted volumetric growth of adults on size-adjusted volumetric growth of juveniles: the proportion of variance explained remained at 49% for both the single and the multiple linear regressions. Interestingly, although monthly growth in somatic tissue mass and in gonadal mass were positively related to monthly growth in shell volume for both juveniles and adults (Table IV), the correlation coefficients were all low (0.28–0.33). The correlation coefficient between monthly growth in shell volume and the sum of somatic growth and gonadal growth for adults was only slightly higher (+0.37).

Our histological observations of the developmental stage of *Mercenaria* gametes

TABLE V

Descriptions of gonad condition from histological sections of Mercenaria *adults*

Sex and collection date		Description
♀	March	All stages of oogenesis present, but mature ova rare. Most oocytes still attached to follicle (1° and 2°). Follicles contain 5–9 developing oocytes.
	June	Almost entirely mature ova. Few attached oocytes. Follicles contain 0–4 ova. Follicles appear larger than in March.
	Sept	Similar to March appearance: all stages of oogenesis present. Some mature ova but most oocytes still attached to follicle walls.
	Dec	Majority in early development (oogonia to 1° oocytes). A few mature ova occur in oviduct. Follicles are smaller than in other months and contain 7–11 oocytes.
♂	March	Wide range of spermatogenic stages. Majority in late spermatogenesis or are spermatozoa. Some empty tubules in center but most full of spermatozoa.
	June	Most tubules empty; those containing spermatozoa hold them in the center with few near the tubule walls. Virtually no intermediate spermatogenic stages present. Gonoducts filled with sperm.
	Sept	Very few spermatozoa present; spermatogonia and 1° spermatocytes are most abundant forms. Some tubules undifferentiated. Gonoducts between tubules are empty.
	Dec	Similar to March appearance, with late stages of spermatogenesis dominating, but differs from March in having more full tubules. No sperm in gonoducts.

(Table V) agree with the earlier descriptions made by Loosanoff (1937a,b), Porter (1964), and Keck *et al.* (1975) for adults of both sexes. Females exhibited only a modest amount of gamete development in the December to March period, with oogonia and 1° oocytes in December progressing to 1° and 2° oocytes in March. Mature ova were rare in March but by 19 June in 1982 they had been produced and most had already been released (Table V). Like females, adult males exhibited relatively little change in developmental stage of gametes between December and March, although the condition of the gametes in males was closer to ripeness in both months (as in data of Eversole *et al.*, 1980). By mid June adult males appeared to have largely completed spawning and early development was evident in September. Out of 17 examined, only one juvenile exhibited gametes at all. A single male, collected in September, contained 1° and 2° spermatocytes, whereas gonadal masses in all other juveniles were small and undifferentiated.

DISCUSSION

Our monthly measurements of size-adjusted growth rates of various components of both juvenile and adult *Mercenaria mercenaria* reflect some combination of seasonal variability in food concentrations, physiological influences of temperature variation, and seasonal shifts in the organism's allocation of resources. Juveniles and adults exhibited a similar pattern of strong seasonality in size-adjusted growth of internal shell volume (Fig. 1). The pattern of seasonal change in shell growth rate resembles the typical diatom productivity pattern for temperate seas, with a major peak in early spring (March) and a smaller peak in autumn. However, data on the seasonality of phytoplankton cell concentrations and of phytoplankton production (in g C) from our study site (Thayer, 1971) do not exhibit patterns that even remotely resemble

Mercenaria's shell growth seasonality: cell concentration peaks in December and reaches a minimum in March, while phytoplankton production is greatest in July at a level over four times the March value. Consequently, the role of seasonal variation of food abundance in determining shell growth is unclear.

Temporal correlations among growth rates of various components of an organism can to some degree reflect changes in allocation of resources among those components. However, any such changes in allocation are confounded by temporal variation in the abundance of the resources available to allocate. Because of seasonal variation both in food abundance and in temperature, the total amount of available resources beyond those required to meet resting metabolic needs is certain to vary greatly over months, especially in a seasonally variable system like a North Carolina estuary. The effect of seasonal variation in available resources is to create an underlying, large degree of concordance among growth rates of various body components, even if allocation among components is changing seasonally. This is reflected in the positive relationships exhibited in all the correlations between pairs of size-adjusted growth rates (Table IV).

The tendency for *Mercenaria* to grow simultaneously in shell volume, somatic mass, and gonadal mass was weak and statistically non-significant (Table IV), despite the strong likelihood of large seasonal variation in available resources. Concordance in growth rate was strongest for adult soma and gonads (Fig. 3), which helps explain why the addition of information on gonadal growth rates of adults as a second independent variable did not explain any of the unexplained variance in the regression of monthly juvenile volumetric growth on monthly adult volumetric growth. The weak correlations among growth rates of various components are a consequence of seasonally changing allocations of resources among components or variability in the estimates of growth. Estimates of growth vary because individuals differ in timing and amount of growth, and because the necessarily sacrificial technique that we were forced to use to estimate growth rates of soma and gonads introduced more variance in the growth estimates than would have appeared if the same individual animals could have been followed. Nonetheless, the weak correlations among size-adjusted growth rates of various components of *Mercenaria* resemble data collected by Kautsky (1982) and Hilbish (1986) on *Mytilis edulis*, showing decoupled seasonal patterns of growth in shell and soft tissues. Seasonal shifts in allocation of resources may prove to be quite general among bivalve molluscs.

The most surprising lack of concordance in any of the seasonal growth patterns in our data is the large increase in the ratio of juvenile to adult shell growth during December and January (Fig. 2). We propose three possible explanations: (1) the stress of cold temperatures affects the size classes differentially, (2) juveniles utilize a winter food source that is not also available to adults, or (3) adults are diverting resources to processes other than shell growth during those two months.

The first potential explanation based upon size-dependent physiological effects of temperature does not seem likely to account for the seasonal anomaly in shell growth because the timing of the anomalous growth does not coincide with the temperature minimum. Broom and Mason (1978) argue on theoretical grounds that larger individual poikilotherms have higher resting metabolic requirements than smaller ones so that as temperature falls large animals have a harder time harvesting sufficient resources to achieve net growth than smaller individuals. This explanation was suggested to explain why queen scallops grew during their first winter of life but not in subsequent ones (Broom and Mason, 1978). The problem with this explanation for our *Mercenaria* data lies in the timing of the growth anomaly: the two months of relatively low adult growth were not the two coldest months. Water temperatures in February and March were substantially colder than in December during 1982 (Fig. 2). To invoke the temperature-dependent physiology explanation, one would have to assume that food resources were much more abundant for *Mercenaria* in February and March and thereby counteracted the effects of low temperature. There are no data on food abundance to support this necessary assumption, but this remains a possible explanation.

The possibility that juveniles may exploit a food source in December and January that is not also utilized by adult *Mercenaria* is difficult to evaluate. A review of filtration efficiencies of suspension-feeding bivalve molluscs (Vahl, 1973) suggests that suspension-feeding bivalve molluscs tend to share a common curve relating particle size to retention efficiency: all particles greater than about 1–2 μm are retained with nearly 100% efficiency by the bivalve gill for bivalves varying over a range of sizes. This implies that it is unlikely that *Mercenaria* juveniles possess a unique food source in December and January in North Carolina. Nevertheless, in the absence of hard evidence, this explanation for the seasonal growth divergence of the two size classes cannot be totally discounted.

The most likely explanation for the anomalously low growth of *Mercenaria* adults relative to juveniles in December and January relates to differences in resource allocation. Although *Mercenaria* within the size class that we term juvenile can actually produce some ripe (male) gametes (Loosanoff, 1937b), the amounts produced are negligible even relative to the small body size (Bricelj and Malouf, 1980; Peterson, 1983). Consequently, a major way in which juveniles differ from adults is in their minimal allocation of harvested resources to reproduction. Because bivalve molluscs presumably store energy and nutrients prior to gametogenesis (Giese, 1959; Bayne, 1976; Gabbott, 1976; Barber and Blake, 1981), the shortfall in adult shell growth in December and January is probably best explained by a large diversion of resources to energy and nutrient reserves in preparation for the tremendous burst of gametogenesis to follow in early spring (Fig. 3, Table V). We hypothesize a phase of nutrient storage during early gametogenesis because the inferred seasonal increase in adult allocation of resources to storage for reproduction during 19 November to 19 January is not evident in gonadal mass (Fig. 3) or histological sections (Table V), the two traditional methods of inferring reproductive processes (*e.g.*, Sastry, 1966). Recent biochemical techniques of inferring energy and nutrient storage (*e.g.*, Barber and Blake, 1985) are necessary to test further our suggestion that *Mercenaria* adults use the two winter months to accumulate energy and/or nutrient reserves for the upcoming demands of gametogenesis. This process also represents an alternative explanation for the similar seasonal growth pattern of queen scallops of different sizes observed by Broom and Mason (1978).

Life-history theory is generally concerned with the optimal allocation of resources between competing demands for those resources, primarily growth and reproduction (Williams, 1966; Gadgil and Bossert, 1970; Stearns, 1976). Testing specific theories in life-history evolution often requires estimates of either the timing of or relative allocation of resources to reproduction (*e.g.*, Charlesworth, 1980; Caswell, 1982). If the failure of adult *Mercenaria* to grow in shell volume during December and January at the rate predicted from observed juvenile growth rates and the 10-month ratio of adult-to-juvenile shell growth rates is a consequence of seasonal change in the quantitative allocation of resources by adult *Mercenaria*, this result has implications for the formulation and testing of life-history theory. First, our results imply that *Mercenaria* like some other marine invertebrates (see Sastry, 1979) has the capacity to alter its allocation of resources between growth and reproduction not only through ontogeny but also through the seasons as an adult. Second, our results imply that detection of the timing and amount of resource allocation to reproduction is a difficult task (Vahl, 1981), probably requiring use of energetic (*e.g.,* Reznick, 1983) or biochemical (*e.g.,* Mann, 1978; Sundet and Vahl, 1981; Barber and Blake, 1985) analyses to complement the more traditional examinations of gonad histology and mass growth.

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METAMORPHOSIS OF *STICHOPUS CALIFORNICUS* (ECHINODERMATA: HOLOTHUROIDEA) AND ITS PHYLOGENETIC IMPLICATIONS

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ABSTRACT

Descriptions of holothurian metamorphosis are based on data from the relatively specialized order Apoda. Metamorphosis for a relatively unspecialized aspidochirote, *Stichopus californicus,* is described here. Metamorphosis in *Stichopus* is characterized by the following features: the madreporic vesicle is a calcite secreting syncytium, not a coelom. *Stichopus* has no separate axocoel and no transient axial complex forms during metamorphosis. The buccal podia form from the water vascular ring, not the radii. The axes of bilateral larval symmetry and the pentaradial adult symmetry are congruent; therefore, the secondarily derived symmetry in holothurians is the pentaradial symmetry of the adult, as in the other extant echinoderms. No axial or visceral torsion occurs during metamorphosis. The enclosed ambulacra form in a manner distinct from that of the ophiuroids and echinoids. Perivisceral coelomic pores develop near the end of metamorphosis, and before the definitive anus forms. Assignment of holothurians with echinoids to the subphylum Echinozoa is discussed.

INTRODUCTION

Detailed comparison of metamorphosis among the extant echinoderm classes led Bury (1895) to propose phylogenetic relationships between them. However, Bury did not propose a phylogenetic position for the holothurians because information on holothurian metamorphosis was incomplete. Today, the phylogenetic relationships between extant echinoderm classes are still disputed. Hyman (1955) and others (Bather, 1900; Beklemischev, 1969), consider holothurians anatomically simple compared to echinoids, because structures such as pedicellariae or movable spines, an axial complex, and Tiedemann's bodies are lacking, and the histological organization of the gonad is relatively uncomplicated. Comparative anatomists interpret this anatomical simplicity of holothurians as primitive and do not group them with echinoids. Paleontologists, however, place holothurians and echinoids in the subphylum Echinozoa (Fell, 1963; Smith, 1984a; Paul and Smith, 1984). Three major arguments support this assignment: (1) both share a globose shape, supposedly the result of similarities in growth patterns. (2) Both have closed ambulacra, and (3), both share calcification around the esophagus in the echinoid lantern and the holothurian aquapharyngeal bulb (Hyman, 1955; Fell, 1963; Smith, 1984a). Embryologists generally agree with paleontologists that holothurian simplicity is derived, and accept the taxon Echinozoa (MacBride, 1914).

Hyman (1955) and Strathmann (1976) show the importance of comparing em-

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bryological processes only among indirect developers. This is not because these larvae are necessarily more primitive, but because of the difficulty in making detailed anatomical comparisons between different types of larvae, and because direct developers do not undergo catastrophic morphogenic rearrangement at metamorphosis. Metamorphosis is of particular interest in echinoderm phylogeny because of the questions raised by Bury's (1895) analyses. However, analyses of holothurian embryology and metamorphosis have been hindered by the inability to fertilize ova *in vitro* (Horstadius, 1925; Mortensen, 1931). The two monographs that do provide analyses of metamorphosis of the holothurian auricularia both focus on *Labidoplax digitata* (Semon, 1888; Bury, 1895), which had to be collected from the sea causing critical stages to be missed (Bury, 1895). Bury noted the limited applicability of his study of metamorphosis in *Labidoplax digitata,* an apodan lacking a number of features common to most holothurians (Ekman, 1925; Hyman, 1955). Bury suggested that an analysis of metamorphosis of an auricularia from a different family would help determine the phylogenetic relationships of holothurians.

Several unresolved questions concerning holothurian metamorphosis remain, including: (1) does an axocoelic madreporic vesicle develop from the hydrocoel (Bury, 1895)? (2) Is an axial complex formed during metamorphosis (Cuenot, 1948; Erber, 1983a, b)? (3) Are the buccal podia formed from the radii or the circumesophageal water ring (MacBride, 1914)? (4) Is the larval axis of bilateral symmetry distinct from the adult axis of pentaradial symmetry (Beklemischev, 1969)? (5) Does an incomplete torsion of the viscera occur during metamorphosis (Bury, 1895)? (6) Are the ambulacra covered by a neurulation like overfolding as seen in echinoids (Runnstrom and Runnstrom, 1918; Runnstrom, 1927)?

My investigation into purification of the oocyte maturation hormone of *Stichopus californicus* (Smiley and Cloney, 1985; Smiley, 1986), allowed me to induce meiosis in oocytes and to fertilize these ova. Over the past five years I raised numerous larvae through metamorphosis and obtained all the stages necessary to complete an analysis of metamorphosis. Here, I describe the structure of the auricularia and mid-metamorphic larvae of *Stichopus californicus,* analyze the process of metamorphosis, and answer the questions raised above. Finally, I present arguments that contradict the hypothesis that holothurians and echinoids share a unique ancestor; arguments which are consistent with the hypothesis that holothurian anatomical simplicity is primitive rather than derived.

MATERIALS AND METHODS

Stichopus californicus adults collected by dredging or diving were maintained in aquaria at the Friday Harbor Laboratories of the University of Washington from 1980 through 1985. Ripe ovaries and testes were obtained from late May through early August. Fecund ovarian tubules were ligated, dissected free of the animal, and maintained in freshly filtered seawater at ambient seawater table temperatures for up to a day. Ripe testes were kept covered at 4°C. until used.

An extract of the radial nerves of *Stichopus californicus,* used to induce meiosis (Smiley, 1984; Maruyama, 1985), was prepared as follows. Adults were eviscerated and the muscle layers dissected free from the body wall. The tissue 2 mm on either side of the midline of each longitudinal muscle contained the radial nerve and was excised. This tissue was pooled, heated to 90°C. for 20 minutes, and homogenized in a blender. The mixture was acidified with acetic acid to a final concentration of 0.1 *M,* then rehomogenized and centrifuged at 18,800 × *g* for 20 minutes. The supernatant was pooled, frozen, and lyophilized. Ten mg/ml of the dry lyophilizate was added to culture vessels which contained freshly dissected fecund ovarian tubules. Ovulation and meiosis occurred within four hours after treatment (Smiley, 1984, 1986).

A sperm suspension was made by pipetting 2 mm of testis, in a pasteur pipet, into 10 ml of freshly filtered seawater. A few drops of this suspension was used to fertilize 100 ml of oocytes immediately after germinal vesicle breakdown. Adding sperm to ova after first polar body formation resulted in elevated levels of polyspermy. Immotile sperm were activated by adding aqueous NH_4Cl to a final concentration of 7–10 m*M*.

Embryogenesis in *Stichopus californicus* follows the pattern described for *Stichopus tremulus* (Holland, 1981). The larvae begin gastrulation about two days after fertilization depending on temperature. About 12 to 18 hours after gastrulation, the mouth has formed and the larvae begin to feed. The larvae were fed with a mixture of the unicellular algae *Dunaliella tertiolecta, Pavlova lutheri,* and *Isochrysis galbana,* grown in 'Algo-Grow' medium (Carolina Biological Supply Co.). Larvae fed a monoculture of any of these algae did not develop well and few completed metamorphosis. Larvae were fed each morning, and the culture water changed each evening with freshly filtered seawater. Growth to competence for metamorphosis took from 18 days to 5 weeks depending on the seawater temperature, the feeding regime, and the source of the eggs.

Larvae were processed for light and transmission electron microscopy by methods previously described (Smiley and Cloney, 1985). Specimens for scanning electron microscopy were fixed in 2% osmium tetroxide in filtered seawater, dehydrated with ethanol and dimethoxypropane. They were dried at the critical point with carbon dioxide, coated with gold, and examined on a JEOL scanning electron microscope. Whole mounts of larvae were made according to Cavey and Cloney (1973).

RESULTS

Metamorphosis of *Stichopus californicus* is quite similar to that of *Labidoplax digitata* (Semon, 1888; Bury, 1895). I define metamorphosis as the transformation of the late larva to the juvenile. Metamorphosis ends when the presumptive adult tissues are in their definitive position. The transformation of the holothurian body during metamorphosis involves morphogenic, histogenic, and organogenic changes, similar to those described for ascidians (Cloney, 1978). Table I summarizes the events of *Stichopus californicus* metamorphosis in temporal order. Because I discuss its phylogenetic implications, I do not describe *Stichopus* metamorphosis in strict temporal order.

Structure of the Stichopus californicus *late auricularia*

The *Stichopus* late auricularia is about a millimeter long and a third of a millimeter wide, with considerable variability between individuals. The body has a complex shape consisting of an oral hood that covers the anterior mid-ventral oral cavity and an anal hood that covers the posterior mid-ventral stomach (Fig. 1). Seen from the side, the oral and anal hoods extend outward from the ventral surface (Fig. 2). The dorsal surface is smooth and has a low convex curvature. Along the margins of the body are small extensions referred to as 'arms'. A continuous strand of ciliated epidermal cells, the ciliated band, lies on the most oblique margin of the body, extending out along the arms. This is the locomotory and feeding organ. The sagittal plane divides the larva into bilaterally symmetric halves, and the frontal plane divides dorsal and ventral surfaces. I term the intersection of these planes the larval axis.

TABLE I

Events in the metamorphosis of Stichopus californicus

Morphogenic metamorphosis 0–4 hours

1. Rapid growth of hydrocoel, formation of anlage of buccal podia, radial vessels, and Polian vesicle.
2. Formation of madreporic vesicle, onset of secretion of madreporic crystal.
3. Change in shape of larva.
4. Reduction in size of larva.
5. Breakup of ciliated band, formation of transverse ciliated rings.
6. Rapid growth of hydrocoel around dorsal side of esophagus.
7. Transient leftwards movement of the opening of oral cavity.
8. Initiation of histolysis of the rectum.
9. Enlargement of coelomic lining of buccal podia.
10. Fusion of left and right ends of hydrocoel at larval mid-ventral line.
11. Protrusion of buccal podia into the oral cavity.

Histogenic metamorphosis 4–24 hours

12. Continued reduction in size.
13. Growth of radial vessels and Polian vesicle.
14. Formation of hyponeural coelom from left and right anterior ends of the somatocoels.
15. Rapid growth of circumoral and radial nervous tissue.
16. Cavitation of tissues superficial to radial nerves to form the epineural sinuses.
17. Movement of opening of oral cavity to anterior end of body.
18. Growth and opening of perivisceral coelomic pore.
19. Migration of madreporic vesicle inward.
20. Fusion of left and right somatocoels.
21. Settlement.

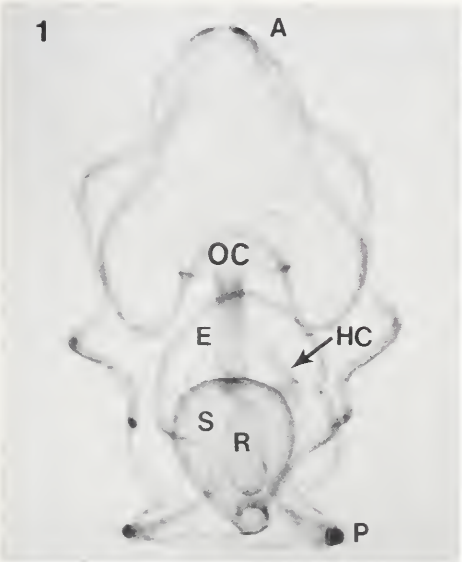
Post-settlement histogenic metamorphosis greater than 24 hours

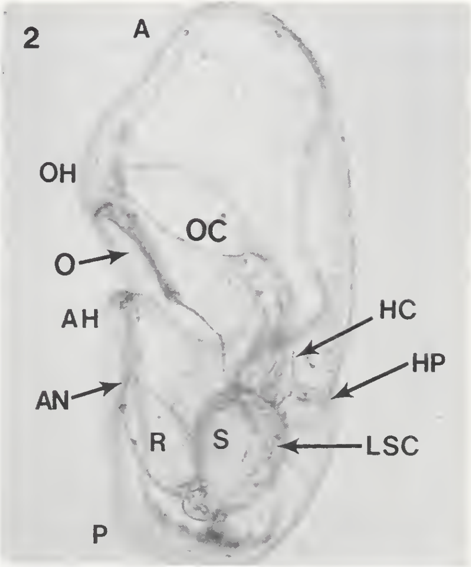
22. Formation of definitive anus.
23. Histolysis of hydropore and distal hydroporic canal.
24. Initiation of spiculogenesis.
25. Formation of somatic podia.

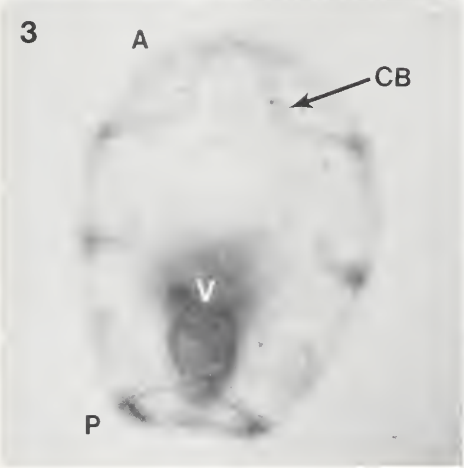
The larval oral cavity leads posteriorly to the mouth and a muscular esophagus connected to the large spherical stomach by a sphincter valve. The intestine and rectum run anteriorly within the anal hood to the anus from the posterior end of the stomach (Fig. 1). The larva has a left and right somatocoel adjacent to the stomach. The hydrocoel is located on the left side of the larva anterior to the left somatocoel and adjacent to the esophageal stomach juncture (Fig. 2). In the early auricularia the hydrocoel is connected to the left somatocoel, but this connection is broken before the onset of metamorphosis. The hydroporic canal runs from the hydrocoel to the hydropore. The hydropore is located just to the left of the mid-dorsal line at about the level of the esophageal stomach juncture. No adult rudiment occurs in this (Fig. 1, 2), or in any other holothurian auricularia that has been studied (Selenka, 1876; Bury, 1895; Mortensen, 1931). A single calcareous 'body' ossicle is found in the lower left arm. This crystalline structure is surrounded by a thin cellular layer.

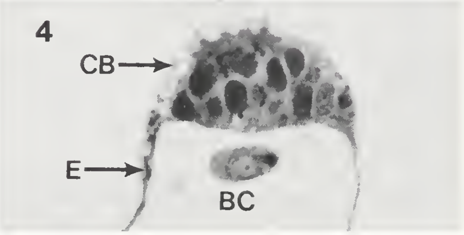
Metamorphosis: general aspects

Metamorphosis will occur within 24 hours when evaginations appear along the surface of the hydrocoel (Fig. 10). Metamorphosis can be divided into two phases. The *morphogenic* phase is relatively rapid, it is completed in about 4 hours, and re-









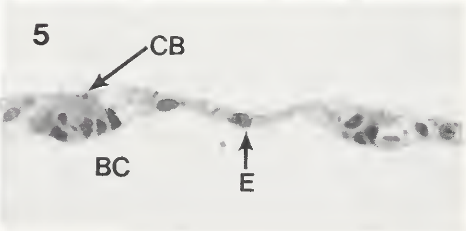


FIGURE 1. A late auricularia of *Stichopus californicus*. A = anterior, E = esophagus, HC = hydrocoel, OC = oral cavity, P = posterior, R = intestine and rectum, S = stomach. Mag. 225×.

FIGURE 2. Left lateral view of an earlier auricularia. A = anterior, AH = anal hood, AN = anus, HC = hydrocoel, HP = hydropore, LSC = left somatocoel, OC = oral cavity, OH = oral hood, O = opening of oral cavity, P = posterior, R = intestine and rectum, S = stomach. Mag. 220×.

FIGURE 3. A mid-metamorphic *Stichopus californicus* larva. A = anterior, CB = ciliated band, P = posterior, V = viscera. Mag. 220×.

FIGURE 4. Section through the ciliated band and epidermis of a late auricularia. BC = blastocoel, CB = ciliated band, E = epidermis. Mag. 970×.

FIGURE 5. Section through ciliated bands and the epidermis of a mid-metamorphic larva. BC = blastocoel, CB = ciliated band, E = epidermis. Mag. 700×.

sults in the formation of the *mid-metamorphic* larva (Fig. 3). Most of the morphogenesis is complete when the larva has assumed a spherical shape at its anterior end. This change in shape is correlated with a noticable thickening of the epidermis (Figs. 4, 5).

The second phase of metamorphosis, the *histogenic* and *organogenic* phase is slower, requiring about 24 hours to complete, and transforms the mid-metamorphic larva to the *pelagic juvenile* (Fig. 14). I have avoided using the term doliolaria for this stage because it is equally applied to the directly developing larvae of holothurians and crinoids as well as the post-metamorphic stage of indirectly developing holothurians. The entire process of metamorphosis is continuous. The stages described here are transitory, grade into one another, and are primarily used for convenience.

Metamorphic transitions during the morphogenic phase

Changes in shape and size. The most obvious difference between the auricularia and the mid-metamorphic larva is the loss of the complex shape (Figs. 3, 7). This change begins at the posterior end of the larva and progresses anteriorly. It is a rapid change, often taking less than an hour to accomplish, and it results in the nearly spherical early mid-metamorphic larva. The transition includes the release of the deformation of the epidermis that contributed to the complex shape of the auricularia (Figs. 1, 3). Associated with this, the uniformly distributed fibers in the blastocoelic connective tissue of the auricularia (Fig. 6) are noticably more dense than in the mid-metamorphic larva (Fig. 7). The late mid-metamorphic larva is approximately two-thirds the length and half the width of the auricularia. The change in size of the auricularia also begins at the posterior end of the larva and gradually makes its way forward. This shrinkage in size is continuous throughout metamorphosis.

Breakup of the ciliated band. Concomitant with the changes in shape and size of the larva is the rearrangement of the ciliated band (Figs. 1, 3). The ciliated band forms incomplete transverse rings around the larva during morphogenic metamorphosis. There are breaks in the rings at the mid-ventral and mid-dorsal lines (Fig. 14). The formation of these rings begins at the posterior end and progresses anteriorly until at the completion of metamorphosis there is a total of five rings. The fourth ring maintains a distinct looped shape until late in metamorphosis when the mid-ventral loop finally separates off and becomes the fifth, most anterior ring (Fig. 3).

Torsion, and the axes of larva and adult. The larval axis, the line of intersection between the sagittal and dorso-ventral planes of the auricularia larva, is identical to the anterior-posterior axis of the mid-metamorphic larva, the pelagic juvenile, and with the anterior-posterior or oral-anal axis of the adult. The larval and adult axial relationships, the pattern of formation of the enterocoel, and the definitive configuration of the larval coeloms for four of the extant echinoderm classes is depicted in Figure 16. Because no extant crinoid has an indirectly developing larva, this class is excluded. Since the axis of symmetry of the larva and juvenile are the same in *Stichopus,* no axial torsion (the twisting of the animal's functional axis) occurs in metamorphosis. Neither does *Stichopus* undergo a visceral torsion (the condition particularly pronounced in ophiuroids, where the viscera twist during metamorphosis so that the line of fusion of the somatocoels is perpendicular to the oral-aboral adult axis) (MacBride, 1914; Hyman, 1955).

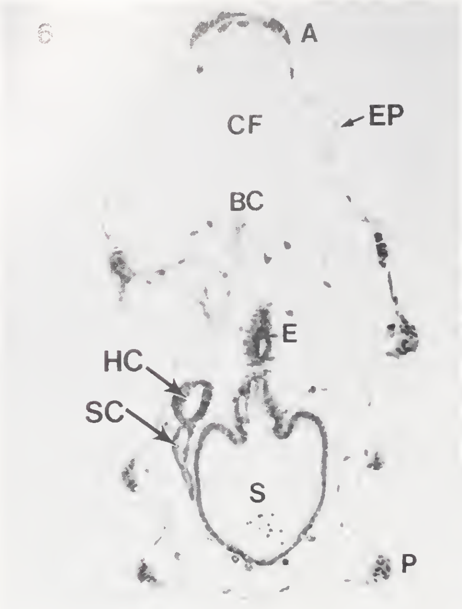
The oral cavity. The opening to the oral cavity of the *Stichopus* larva becomes slightly displaced to the left with respect to the larval axis during the first phase of metamorphosis. This change is transitory; the position of the opening relative to the axis is restored as the larva continues to shrink. The shape of the oral cavity also changes during metamorphosis, due to differential changes in the topology of the larval epidermis. Previously, the oral cavity was called the "vestibule," and the opening of the oral cavity, the "mouth." These terms imply homology with the developing rudiment of echinoids and are not used here. The true mouth of both the auricularia and the mid-metamorphic larva is situated at the bottom of the oral cavity, at the anterior end of the larval esophagus (Fig. 1). The mouth and the oral cavity are present in the auricularia and do not form by invagination as Bury (1895) suggested. The appearance of invagination is produced by changes in the epidermis that occur during metamorphosis. No rudiment develops near the oral cavity, or anywhere else, in this holothurian. When metamorphosis is complete, the oral cavity becomes the oral sheath surrounding the buccal podia of the juvenile (Fig. 15).

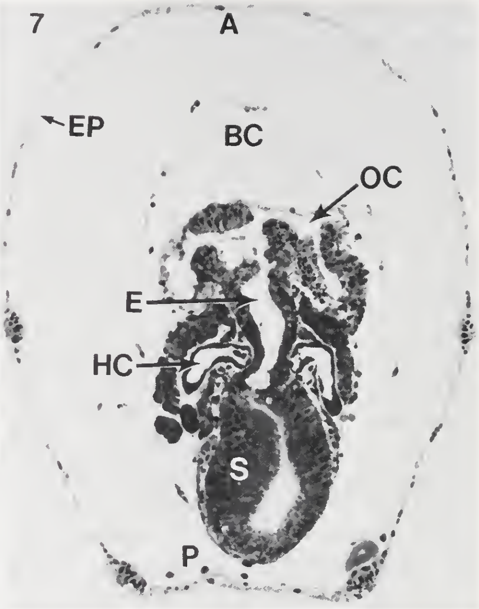
Changes in the larval gut. During the morphogenic phase in *Stichopus californicus,* the larval intestine and rectum (Fig. 1) lose their tubular form and begin to histolyze (Figs. 18, 19). The larval anus initially remains open during morphogenic metamorphosis (Fig. 8), but it soon closes. The definitive intestine begins to form at the posterior end of the stomach during the histogenic phase of metamorphosis, but the definitive anus is not fully formed before settlement. The larval stomach changes considerably during the morphogenic phase (Figs. 6, 7). The stomach wall becomes thicker and the entire structure shrinks to a fraction of its former size. No marked histolysis occurs other than in the tissues of the intestine, rectum, and anus.

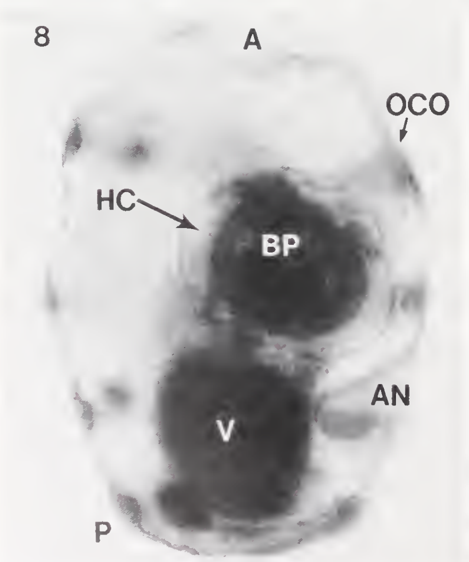
Development of the hydrocoel and water vascular system. Just prior to the onset of metamorphosis five distinct lobes or evaginations develop on the anterior surface of the hydrocoel (Fig. 10). The lobes greatly enlarge during the first stage of metamorphosis (Figs. 8, 9), but remain on the anterior surface of the hydrocoel. The evaginations can be followed during metamorphosis (Figs. 9, 17), as they become the coelomic lining of the primary buccal podia. Once they have grown to just beneath the epidermis of the oral cavity, they appear to induce the epidermis of the oral cavity to grow out around them (Figs. 9, 17). Prior to settlement, the buccal podia can protrude from the oral cavity (Fig. 15). The juvenile's buccal podia attach to the substratum during settlement in *Stichopus.* At no time during the ontogeny of the buccal podia is their hydrocoelic lining directly connected to an ambulacral water vascular canal.

The hydrocoel itself also enlarges rapidly during this morphogenic stage, principally by division of the coelomic epithelial cells. It grows around the esophagus (Fig. 9) from the left to the right side of the larva, to meet itself near the larval mid-ventral line. When its left and right ends have fused, the hydrocoel becomes the circumesophageal water vascular ring. The five evaginations that will become the ambulacral water vascular radial canals develop on the posterior surface of the hydrocoel, between the evaginations of the buccal podia. The mid-ventral radial water vascular canal of *Stichopus* grows considerably faster than the others (Fig. 18). This growth primarily involves the posterior elongation of the ambulacral water vascular canals, which are superjacent to the developing perivisceral coelomic peritonea and subjacent to the connective tissue compartment of the body wall. At no time are the developing radial canals of the water vascular system close to the epidermis of the larva. An eleventh evagination of the hydrocoel forms the Polian vesicle of the pelagic juvenile.

The madreporic vesicle. The madreporic vesicle (Fig. 13) is spherical, forms around the hydroporic canal in the late auricularia larva, and is an example of a histogenic change associated with the first phase of metamorphosis. The madreporic vesicle surrounds the developing madreporic crystal (Figs. 10, 11), which has the shape of a fine filigree and is composed of calcareous material (Fig. 11), judged by polarizing microscopy. This vesicle is first visible just beneath the larval epidermis, separated from the larval hydrocoel by the length of the hydroporic canal. By the end of the morphogenic phase, the vesicle has withdrawn into the interior of the animal. This inward movement is greater than the diminishment in larval size; consequently, the motion cannot be explained by the reduction in larval size alone. The most proximal portion of the larval hydroporic canal becomes the adult stone canal in *Stichopus*







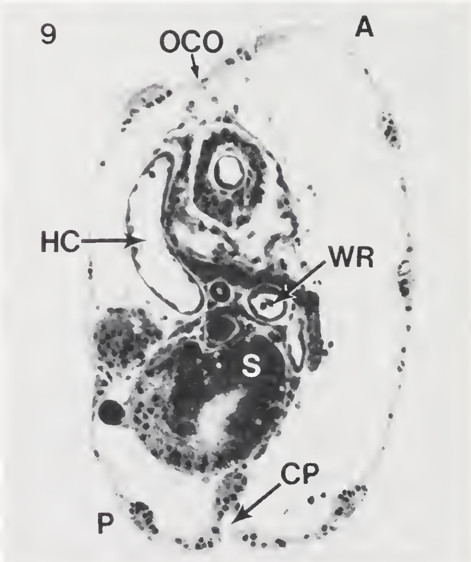


FIGURE 6. Frontal section through a late auricularia. A = anterior, BC = blastocoel, CF = connective tissue fibers, E = esophagus, EP = epidermis, HC = hydrocoel, P = posterior, S = stomach, SC = somatocoel. Mag. 160×.

FIGURE 7. Frontal section through a mid-metamorphic larva. A = anterior, BC = blastocoel, E = esophagus, EP = epidermis, HC = hydrocoel, OC = oral cavity, P = posterior, S = stomach. Mag. 220×.

FIGURE 8. Whole mount of a metamorphosing *Stichopus* larva. A = anterior, AN = closing anus, BP = forming buccal podia, HC = hydrocoelic lining of buccal podia, OCO = opening of oral cavity, P = posterior, V = viscera. Mag. 330×.

FIGURE 9. Saggital section through a late metamorphosing larva. A = anterior, CP = perivisceral coelomic pore, HC = hydrocoel, OCO = opening of oral cavity, P = posterior, S = stomach, WR = water vascular ring canal. Mag. 250×.

californicus. Cilia within the hydropore stop beating prior to settlement and the hydropore closes soon after the larva settles.

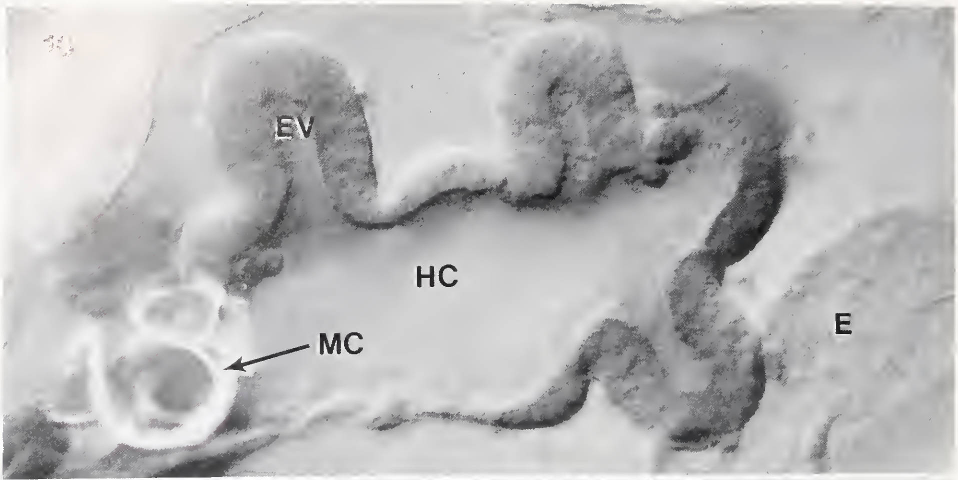
Previous investigators held that the madreporic vesicle was the holothurian axocoel (see Bury, 1895). In *Stichopus californicus*, only a thin cellular layer surrounds each of the fine calcareous filaments of the madreporic crystal (Fig. 13). Transmission electron microscopy reveals that this layer is part of a single cell and not an epithelium (Fig. 20). The perikarya of the cells forming the madreporic vesicle syncytium lie in its center and are morphologically similar to the perikarya of the syncytium which secretes the larval skeleton in echinoids (Gibbins *et al.*, 1969). I found no evidence of intercellular junctions between the syncytium of the madreporic vesicle and the epithelial cells of the hydroporic canal, a condition consistent with the observation that the madreporic vesicle migrates along the hydroporic canal during metamorphosis. The outer surface of the madreporic vesicle is associated with a prominent external lamina, similar to the external lamina that surrounds ossicle secreting syncytia in adults of the holothurian *Leptosynapta clarki* (Stricker, 1985). The structure of the madreporic crystal supports the hypothesis that it is secreted within intracellular vacuoles of the syncytium (Figs. 13, 20), reminiscent of the secretion of the larval skeleton of ophiuroids and echinoids (Okazaki and Inoué, 1976).

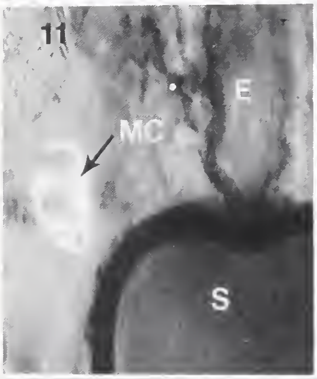
The pelagic juvenile: histogenic metamorphosis

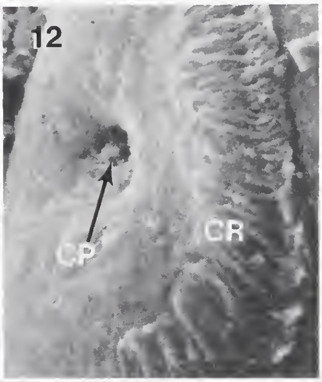
The changes required to make a pelagic juvenile (Fig. 14) from the mid-metamorphic larva are not substantial. The pelagic juvenile is more cylindrical and about half the size of the mid-metamorphic larva by the time of settlement. Upon completion of metamorphosis, the anlagen of all the juvenile tissues are in their appropriate locations. Consequently, most of the changes during this period involve tissue growth (see Table I). The larva continues to shrink during this second phase of metamorphosis. The net result of this shrinkage is the further condensation of the body wall connective tissue compartment (Figs. 7, 9). Growth of the left and right somatocoels, coupled with decrease in the size of the larva, causes the somatocoels to become closely applied to one another while lining the body cavity and surrounding the gut. When the somatocoelic epithelia fuse together, these cavities become the perivisceral coelom. The mesenteries formed in the fusion of the somatocoels are parallel to the larval axis.

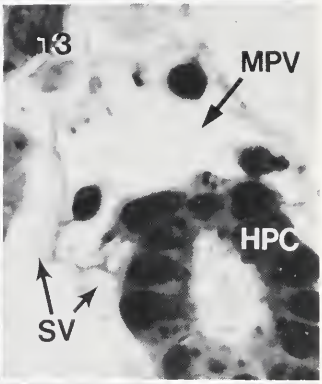
Development of the perivisceral coelomic pore. A pore is first visible in scanning electron micrographs of the posterior surface of the pelagic juvenile (Fig. 12). This is not the anus but a perivisceral coelomic pore (Fig. 9), which is connected to the right somatocoel. The connection to the somatocoel was ascertained only after examination of serial semi-thin cross sections. The single coelomic pore opens before histolysis of the larval rectum is complete, and the definitive anus forms.

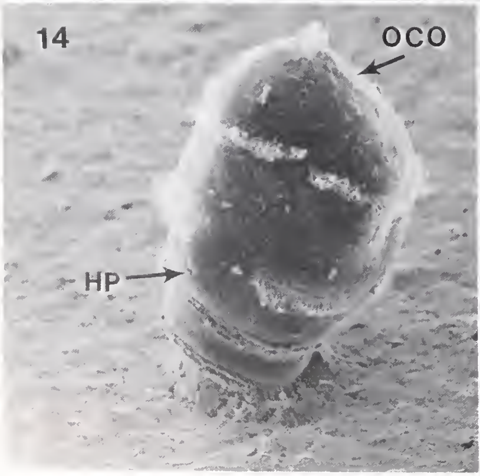
The enclosure of the ambulacra. In the mid-metamorphic *Stichopus* larva, the elongating water vascular radial canals grow posteriorly from the circumesophageal water vascular ring canal to deep within the larva. The radial canals are a considerable distance from the epidermis, separated by the connective tissue compartment of the body wall (Figs. 9, 18). Tissues that will contribute to the radial nerves of the juvenile are superjacent to each elongating radial canal and extend from the circumesophageal nerve ring to near the end of the radial canal (Figs. 18, 21, 22). Superjacent to the anlage of the radial nerve and at a more anterior location, is an area where the epineural sinus first appears (Fig. 22). I found no evidence of an epineural sinus beneath the floor of the oral cavity, and superficial to the circumesophageal nerve ring in any specimens, although it occurs in this position in adults (Hyman, 1955). The epineural











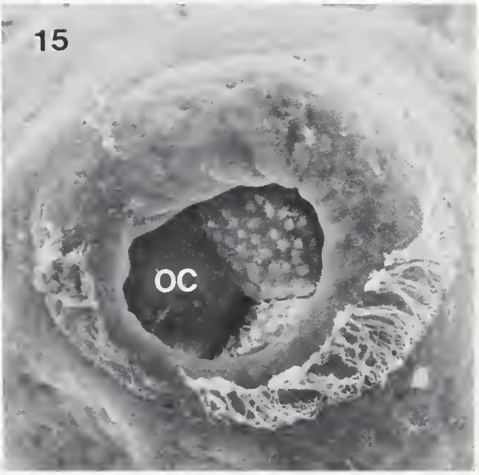


FIGURE 10. Doubly exposed polarizing and differential interference contrast micrograph of the larval hydrocoel and madreporic crystal. E = esophagus, EV = evaginations of the hydrocoel, HC = hydrocoel, MC = madreporic crystal. Mag. 1230×.

FIGURE 11. Doubly exposed polarizing and DIC micrograph of the madreporic crystal surrounding the hydroporic canal. Note that the madreporic crystal has the shape of a fine filigree. E = esophagus, MC = madreporic crystal, S = stomach. Mag. 620×.

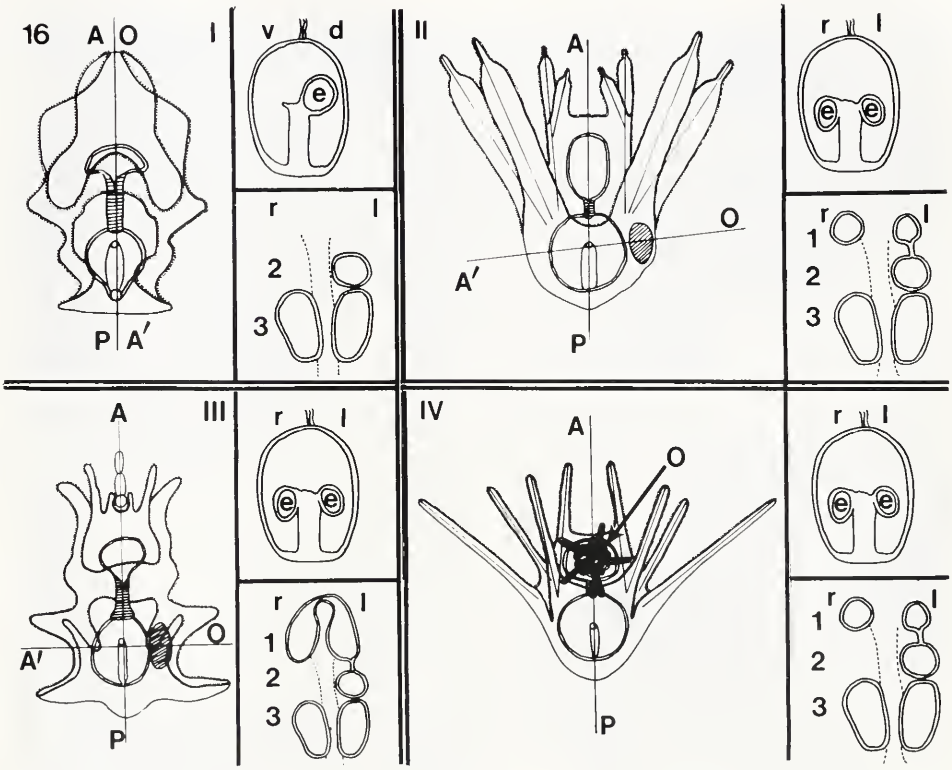


FIGURE 16. Larval and adult axial relationships as well as coelomic configurations at formation and in the definitive larva in four classes of echinoderms. I = Holothurians, II = Echinoids, III = Asteroids, and IV = Ophiuroids. The axis of pentaradial symmetry of ophiuroids is perpendicular to the plane of the page, but also perpendicular to the larval axis. The position of the adult rudiment is indicated by diagonal lines. A—P indicates the larval axis, O—A′ indicates the axis of the adult. r, l = right, left. d, v = dorsal, ventral. e = enterocoelomic vesicle. 1 = Axocoel. 2 = Hydrocoel. 3 = Somatocoel.

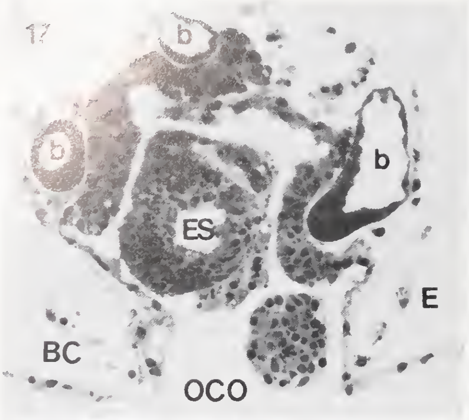
sinus of *Stichopus* appears to be formed by cavitation of tissues superficial to the presumptive radial nerve (Figs. 18, 22). In longitudinal sections through developing ambulacra, the tissue that will form the epineural sinus is visible as a single layer of squamous cells superjacent to the radial nerve anlage. I found no evidence of epineural flaps, nor of their folding over, either along the larval body, or on the floor of the oral cavity. Finally, no indications of the hyponeural sinus were found along the ambulacra, suggesting that this structure develops after metamorphosis.

FIGURE 12. SEM of the posterior of a late metamorphosing larva. CP = perivisceral coelomic pore, CR = posterior most ciliated ring. Mag. 990×.

FIGURE 13. Section through the madreporic vesicle. HPC = hydroporic canal, MPV = madreporic vesicle, SV = secreting vacuoles of the syncytium. Mag. 1750×.

FIGURE 14. SEM of a right rear view of a pelagic juvenile larva of *Stichopus* showing the incomplete transverse ciliated rings. The apparent apical tuft is debris, a preparation artifact. HP = hydropore, OCO = opening of the oral cavity. Mag. 280×.

FIGURE 15. SEM of the opening of the oral cavity of a pelagic juvenile larva, showing the tips of the buccal podia. OC = oral cavity. Mag. 790×.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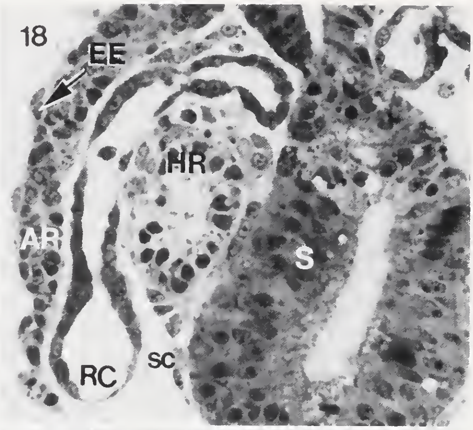
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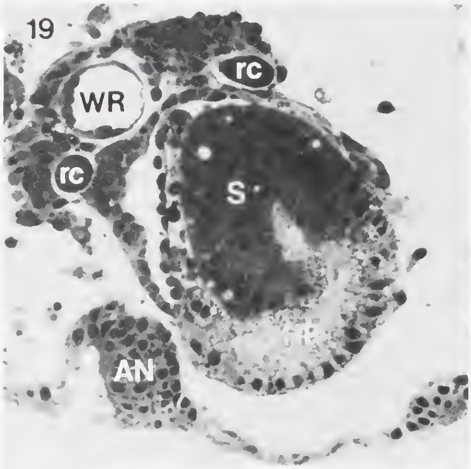
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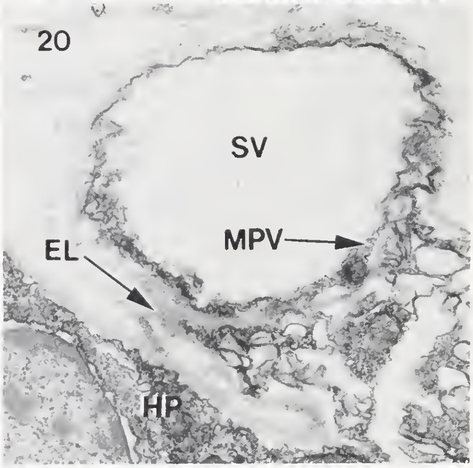
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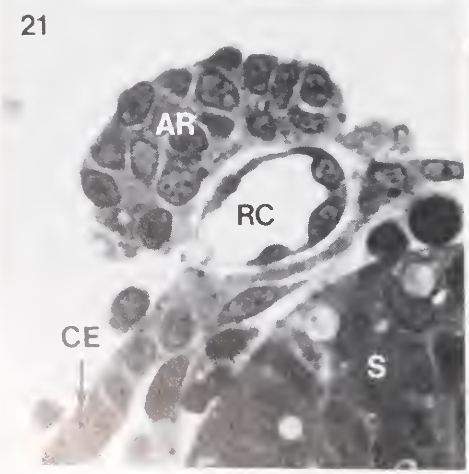

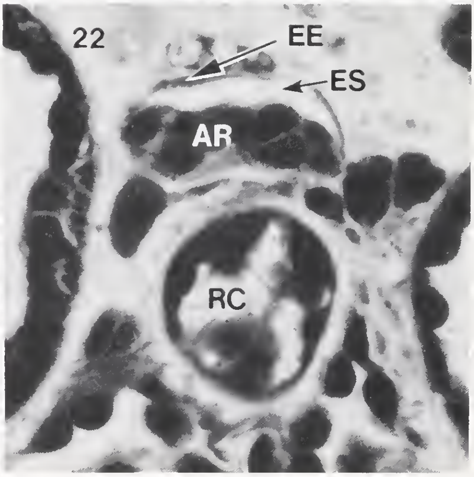
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FIGURE 17. Section through the opening of the oral cavity of a mid-metamorphic larva. The ventral surface is at the bottom of the figure. B = buccal podia coelomic lumen, BC = blastocoel, E = epidermis, ES = esophagus, OCO = opening of oral cavity. Mag. 390×.

FIGURE 18. Section through the visceral region of a metamorphosing *Stichopus* larva. AR = anlage

No axial complex or gonad is formed during metamorphosis

No axial complex forms during metamorphosis in *Stichopus californicus.* The transient presence of this organ in holothurians was suggested by Cuenot (1948). Neither is the connective tissue component of the axial complex, the axial gland (Erber, 1983b), present in newly metamorphosing *Stichopus californicus,* suggesting that this structure originates later in development. There is no primordium of the gonad in newly metamorphosed animals, consistent with reports that the onset of echinoderm gonadogenesis occurs some months after settlement (Nieuwkoop and Sutasurya, 1981).

Discussion

Stichopus californicus metamorphosis follows the general pattern described for *Labidoplax digitata* by Semon (1888) and Bury (1895). This metamorphosis differs from previous accounts in that: (1) the madreporic vesicle, a structure presumed to be an axocoel by Bury (1895), is instead a syncytium. The madreporic crystal is secreted within intracellular vacuoles of this syncytium. (2) No transient axial complex forms during metamorphosis. (3) The coelomic linings of the buccal podia are derived from the circumesophageal water vascular ring canal and not from the radial canals. (4) The axes of bilateral and pentaradial symmetry of the juvenile are congruent, and are identical to the larval axis. (5) There is no torsion of the viscera during metamorphosis of this holothurian. (6) There is no folding over of epineural tissue flaps to enclose the ambulacra in *Stichopus;* the epineural sinus is formed by cavitation of tissue layers superficial to the radial nerves. (7) A perivisceral coelomic pore, which connects the right somatocoel to the sea, forms before the definitive anus, during the time the lower larval gut is being resorbed.

Mechanisms of morphogenic metamorphosis

My analysis of metamorphosis in *Stichopus californicus* suggests two hypotheses concerning the mechanisms controlling the process. First, a decrease in the stiffness of the blastocoelic connective tissue occurs at the onset of the first phase of metamorphosis. This decrease allows the shape of the larva to change from the complex form of the auricularia to the roughly spherical mid-metamorphic larva. Changes in the stiffness of holothurian connective tissue have been documented for a number of species under a variety of conditions (Smith and Greenberg, 1971; Emson and Wilkie, 1980; Motokawa, 1984). While these changes appear to be under nervous control, their biochemical basis is not resolved. If the blastocoelic connective tissue were

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of radial nerve, EE = epineural sinus epithelium, HR = histolyzing intestine and rectum, RC = radial canal, S = stomach, SC = somatocoel. Mag. 650×.

FIGURE 19. Section through histolyzing intestine and rectum of a metamorphosing larva. AN = anus, HR = histolyzing tissue, RC = radial canal, S = stomach, WR = water vascular ring canal. Mag. 440×.

FIGURE 20. TEM through a secreting vacuole of the madreporic vesicle. EL = external lamina, HP = hydroporic canal, MV = madreporic vesicle, SV = secreting vacuole. Mag. 15,330×.

FIGURE 21. Cross section through the posterior region of the elongating mid-ventral radial canal of a metamorphosing larva. AR = anlage of the radial nerve, CE = perivisceral coelomic epithelium, RC = radial canal, S = stomach. Mag. 1570×.

FIGURE 22. Section through a more anterior region of a developing ambulacrum. AR = anlage of the radial nerve, EE = epineural sinus epithelium, ES = epineural sinus, RC = radial canal. Mag. 2100×.

stiff in the auricularia, it would allow the animal to maintain its complex shape with a minimum of energy expended. The complex shape of the auricularia is likely to facilitate feeding and locomotion driven by the ciliated band (Strathmann, 1971). The observation that the blastocoelic connective tissue fibers are more dense in the auricularia than in the mid-metamorphic larva is consistent with this hypothesis.

The second hypothesis is that changes in the shape of epidermal cells contribute to the tension within the epidermis. In this case, epidermal tension works concurrently with the changes in the stiffness of the connective tissue to produce the spherical mid-metamorphic larva. I have documented changes in the shape of the larval epidermal cells, from squamous to low cuboidal, during metamorphosis (Figs. 4, 5). I interpret these changes to be a reflection of the tension within these cells. Continued tension may be responsible for the gradual reduction in size that accompanies the transformation of the mid-metamorphic larva to the pelagic juvenile.

The morphogenic movements which occur in the metamorphoses of asteroids, ophiuroids, and echinoids are exceedingly complex (MacBride, 1914; Hyman, 1955), and cannot be described easily here. Comparison of metamorphic morphogenesis in these animals with that in *Stichopus californicus* shows that there are both fewer movements and less complicated mechanisms required to explain them in holothurians.

Symmetry and the patterns of holothurian metamorphosis

The auricularia larva, like all indirectly developing echinoderm larvae, is distinctly bilateral. The bilateral symmetry probably makes for economy in the control of development, as well as economy in locomotion and feeding (Strathmann, 1971). Like all adult echinoderms, adult holothurians have pentaradial symmetry. The hydrocoel organizes this pentaradial symmetry beginning at metamorphosis, and maintains it during growth through the influence of the radial vessels of the water vascular system.

Holothurians also bear a bilateral symmetry in the adult, a symmetry most evident in the division of the body into a ventral trivium and a dorsal bivium. It is usual, however, to refer to the bilaterality of adult holothurians as a secondary acquisition, underlining the supposedly derived character of the class (Hyman, 1955). A unique difference in holothurian metamorphosis is that there is no axial torsion of the body to a position 90 degrees from the larval axis, as is the case in the asteroids and echinoids (see Fig. 16). Neither is there torsion of the viscera to a position 90 degrees off the larval axis, as is the case in ophiuroids (Bury, 1895). Metamorphic torsion can be observed or inferred in the other classes either from the location of the adult rudiment or from the configuration of the coelomic mesenteries supporting the gut (Bury, 1895; Hyman, 1955).

Bury (1895) interpreted the leftwards motion of the opening of the oral cavity in *Labidoplax* as a slight or vestigial torsion during early metamorphosis, as does Ohshima (1921). I corroborate Bury's observation of this movement in *Stichopus,* but I suggest that the transient leftwards displacement of the opening of the oral cavity is due to the growth of the hydrocoel around the larval esophagus during the first phase of metamorphosis, rather than to an ancestral reminiscence of visceral torsion as proposed by Bury (1895) and elaborated by MacBride (1914). In *S. californicus,* the oral cavity can be followed from the auricularia through to settlement, but Bury (1895) presumed that the oral cavity formed at metamorphosis, and used this observation to support a homology between the echinoids and holothurians. Bury's misinterpretations were probably due to his inability to culture *L. digitata in vitro,* causing him to miss the intervening stages. Not all echinoids have a vestibule (Emlet, pers. comm.), indicating that echinoid development may not be as distinct from that of asteroids as previously thought (MacBride, 1914).

I have found no accounts of the position of the coelomic mesenteries of any holothurian which could be interpreted to reflect a torsion of the viscera about the body axis in metamorphosis. During *Stichopus* metamorphosis, the bilaterality of the juvenile is congruent with, and derived from, the bilaterality of the auricularia. Therefore, the assertion that the bilaterality of holothurians is secondary is incorrect. The symmetry acquired secondarily in holothurians, as in all echinoderms, is the pentaradial symmetry of the adult.

The shift of the functional axis during metamorphosis of asteroids, ophiuroids, and echinoids entails tremendous changes. These changes are correlated with the presence of an adult rudiment on the larva, which dominates the development of the post-metamorphic juvenile; the greater preponderance of the larval tissues being lost or phagocytized (Chia and Burke, 1978). The ingression of strictly adult tissues into the larval body often indicates that temporal compression in development has occurred (Gould, 1977). No axial transformations occur during holothurian development, either in the metamorphosis of indirectly developing species or in the growth and settlement of direct developers (Semon, 1888; Bury, 1895; Ohshima, 1921; Inaba, 1930). Nor are there any reports of the development of a rudiment on the larvae of indirectly developing holothurians (Selenka, 1876; Semon, 1888; Bury, 1895; Mortensen, 1931; Rustad, 1940). In the metamorphosis of *Stichopus,* only the larval intestine, rectum, and anus are resorbed, and the majority of all holothurian larval tissues are directly incorporated into the juvenile (Semon, 1888; Bury, 1895). The absence of an adult rudiment on the holothurian auricularia larva indicates that there has been no massive temporal compression of development. Therefore, when we compare the patterns of metamorphosis in holothurians with those in asteroids, ophiuroids, and echinoids, we contrast untorted, simple, and continuous morphogenic movements showing no evidence of developmental compression, with torted, complex, and discontinuous morphogenic movements where there is good evidence for temporal compression.

These arguments support the conclusion that the patterns of holothurian metamorphosis are considerably more simple than the patterns described for asteroids, ophiuroids, and echinoids. The available data are not consistent with the hypothesis that the simplicity of holothurian metamorphosis is derived. If this were the case we would expect to find vestiges of a more complex mechanism of morphogenic movements, of axial transformation, or of an adult rudiment in holothurian metamorphosis. The hypothesis that the simplicity of holothurian metamorphosis might be derived and yet leave virtually no trace, does not offer explicit testable predictions. I do not believe that such extraordinary compression of development could occur and be undetectable. Finally, the patterns of metamorphosis in *Stichopus* do not support the hypothesis that holothurians and echinoids are closely related.

The internal madreporite and coelomic pores

In *Stichopus californicus,* the madreporite is internal and is the terminus of the stone canal. This is also the case in most other holothurians including those groups traditionally thought to represent the ancestral holothurian stock, the aspidochirotes and dendrochirotes (Eckman, 1925; Hyman, 1955). There is some controversy as to the definitive location of the madreporite in holothurians (Erber, 1983a) because in the elasipods the madreporite is lodged in the body wall and in some molpadids the larval hydropore fails to close (Hyman, 1955). Most authorities acknowledge that the condition in these two orders is derived (Ekman, 1925; Hyman, 1955). There is little question, however, that the internal madreporite in holothurians functions to allow passage of water vascular and coelomic fluids (Nichols, 1969). Crinoids, as far as is known, have neither a madreporite nor a madreporic vesicle, but the ends of their many stone canals hang freely in the perivisceral coelom, and it is likely that fluids pass through the open ends of the stone canals (Bather, 1900). The madreporite in asteroids, ophiuroids, and echinoids is external and also functions in the passage of fluid (Hyman, 1955). The inner surface of the madreporite is derived from the right axocoel in these echinoderms and it surrounds the terminus of the stone canal (Hyman, 1955). Only a few extinct echinoderms possessed an external madreporite, including all the ophiocystioids, although many had a hydropore as adults (Bather, 1900; Hyman, 1955; Nichols, 1969). The presence of an external madreporite in the ophiocystioids, asteroids, ophiuroids, and echinoids, and its absence in all other echinoderm classes suggests that its presence is a derived condition.

Prior to the formation of the definitive anus in *Stichopus,* a coelomic pore develops which connects the right somatocoel with the exterior. The early development of this pore suggests a functional significance in the adult. These pores have recently been described for adult *Stichopus californicus* (Shinn, 1985a), and their fine structure noted (Shinn, 1985b). While there is no evidence that the other eleutherozoans have coelomoducts, extant and extinct pelmatozoans do possess pores which may have functioned in conveying seawater to the perivisceral coelom. Crinoids have ciliated pores which are perivisceral coelomoducts (Hyman, 1955), and many extinct echinoderms also had respiratory coelomic pores passing through the body wall (Bather, 1900). This suggests that pores connecting the perivisceral coelom with the sea may be primitive in echinoderms. This conclusion is further strengthened by the observation that coelomic pores are correlated with the absence of an external madreporite. In summary, these arguments contradict the hypothesis that echinoids and holothurians shared a common ancestor, and supports the hypothesis that the presence of an internal madreporite in holothurians is primitive rather than derived.

Closed ambulacra

The ambulacra of holothurians are covered by the body wall, and an epineural sinus is found superficial to the radial nerves (Hyman, 1955). This morphology is similar to that of ophiuroids and echinoids, but distinct from the naked ambulacra of the crinoids and asteroids (Hyman, 1955). The presence of closed ambulacra has been used to argue an evolutionary relationship between the holothurians and the echinoids (Fell, 1963; Smith, 1984a). However, in echinoids and ophiuroids, the ambulacra are closed by the folding over of epineural flaps of tissue in a manner reminiscent of the neurulation of vertebrate embryos (MacBride, 1914). In *Stichopus californicus,* the ambulacral epineural sinus forms by cavitation of the tissue layers superficial to the radial nerves. I found no evidence of a neurulation-like event either at the circumesophageal nerve ring or along the radial nerves. Hyman (1955) claimed that Runnstrom and Runnstrom (1918) and Runnstrom (1927) reported that epineural flap overfolding enclosed the epineural sinus of several holothurians. In fact, these investigators only suggest this process as a likely possibility; they did not observe such an event (Runnstrom and Runnstrom, 1918; Runnstrom, 1927).

I suggest that one reason for the epineural overfolding of ectodermal tissue flaps during the closure of echinoid ambulacra may be to invaginate ectodermally derived tissues to a sub-epithelial location, a process required as a consequence of the radical metamorphosis in these animals. The ectodermal tissues then could be recruited to form the circumesophageal and radial nerves. Holothurians already have nerve cells in the area of the mouth as reported by Burke *et al.* (1986). Thus, an invagination may be unnecessary in this class. Since the epineural sinus of holothurians is formed by a mechanism different from that found in the echinoids and ophiuroids, closed ambulacra do not necessarily indicate a close evolutionary relationship between these classes and the holothurians (Hyman, 1955; Fell, 1963; Smith, 1984a). Nichols (1967) suggested that closed ambulacra may have arisen more than once in the echinoderms. The evidence from *Stichopus californicus* supports this suggestion. The different modes of enclosure of the ambulacra in holothurians and echinoids directly contradict the hypothesis that holothurians and echinoids share a common ancestor. However, we cannot infer if the condition in holothurians is primitive or derived from the available evidence.

Coelomic organization

The notion of a bilaterally paired, tripartite coelomic organization of larvae has been deeply integrated into phylogenetic studies of echinoderms (Bather, 1900; Hyman, 1955). The homologies of both the water vascular and perivisceral coeloms in the extant classes pose few problems (Bather, 1900; MacBride, 1914; Hyman, 1955). But, it is necessary to explain the relationship between the single anterior coelomic compartment of holothurians and the several anterior coelomic compartments of the asteroids, ophiuroids, and echinoids. In nearly all holothurians the original enterocoelic vesicle (Fig. 16), which gives rise to the anterior coelom, is single and arises dorsally (Selenka, 1876; Ohshima, 1921; Inaba, 1930; Rustad, 1940). In contrast, the enterocoelic vesicles of asteroids, ophiuroids, and echinoids are paired and arise laterally (MacBride, 1914; Hyman, 1955).

The madreporic vesicle of *Stichopus,* a structure reported to be an axocoel in other holothurians (Bury, 1895), is a calcite secreting syncytium and not an epithelium, which the definition of a putative coelomic tissue requires (Hyman, 1955). Since the madreporic vesicle is a syncytium in *Stichopus,* it is not homologous with the axocoelic coelom of asteroids, ophiuroids, and echinoids. Therefore, I conclude that there is no separate axocoel in this animal. My interpretation argues that Bury was incorrect in asserting that the madreporic vesicle of *Labidoplax* was the holothurian axocoel.

The madreporic vesicle of *Stichopus californicus* lies around the hydroporic canal, close to the body wall. Since there are no intercellular junctions between the cells of the madreporic vesicle and those of the hydroporic canal, and since calcite secreting syncytia in other echinoderms are formed from mesenchyme (Okazaki and Inoué, 1976; Gibbins *et al.,* 1969), I suggest that the madreporic vesicle is also formed from mesenchyme. Mesenchyme cells in holothurians arise from the vegetal plate, the archenteron and the enterocoelomic vesicle (Selenka, 1876; Semon, 1888; Bury, 1895; Rustad, 1940). If the madreporic vesicle was an epithelium formed from mesenchyme, it might be a coelom, but it still would not be homologous with the axocoel of the asteroids, ophiuroids, and echinoids. The madreporic vesicle in these classes arises from the axocoelic coelom (Bury, 1895; MacBride, 1914; Hyman, 1955), which divides off from the hydrocoel during development (MacBride, 1914; Hyman, 1955), but remains connected to it through metamorphosis (Fig. 16). My observations indicate that the separation of these anterior coeloms by Bury (1895) may have obscured some of the evolutionary relationships in the phylum. Hyman (1955) recognized this problem and referred to the combination of axocoel and hydrocoel as an *axohydrocoel* to underline their connection.

The axial sinus of asteroids, ophiuroids, echinoids, and crinoids is derived from the axocoel (Bury, 1895; MacBride, 1914; Hyman, 1955). In asteroids, ophiuroids, and echinoids, the axial sinus is required for the establishment of the genital rachis, the progenitor of the gonad (Hyman, 1955), but, no genital rachis forms in holothurians (Hyman, 1955; Smiley and Cloney, 1985). The axial sinus is a prominent coelomic component of the axial complex, which also contains a connective tissue component, the axial gland, (Hyman, 1955; Erber, 1983a, b). Erber (1983b) has shown that an axial gland is present near the dorsal hemal structures in several adult holothurians. I have found no evidence of the axial gland in metamorphosing *Stichopus,* indicating that its development must occur after metamorphosis is complete. I have found no evidence for even a transient existence of an axial sinus in *Stichopus.* Since the axial gland forms in crinoids in the absence of an association with the axial sinus, it is reasonable to assume that the condition in *Stichopus* is similar. Erber (1983a, b) however, concludes that a vestigial axial sinus is represented by the madreporic vesicle. The evidence from *Stichopus* does not support this hypothesis. It may be that the perivisceral coelom encloses the madreporite of adult holothurians, or the peritoneum surrounding the madreporite may be a secondary coelomic derivative like the peripharyngeal or perianal coeloms (Hyman, 1955).

With these data, I examine the question of whether the undivided axohydrocoel in holothurians is primitive or derived. Although we cannot directly test these hypotheses, we can make predictions of structure and function based upon them. First, if the undivided axohydrocoel is primitive, we can predict that holothurians would lack structures such as a pentaradial gonad or a transitory axial sinus at metamorphosis; structures that are the direct result of an inductive influence by the separate axocoel in the asteroids, ophiuroids, and echinoids. Further, we can predict that those subsidiary functions subsumed by the axocoel in asteroids, ophiuroids, and echinoids, such as formation of the distal portion of the stone canal and attachment of the larva at metamorphosis, would be subsumed by the axohydrocoel alone in holothurians. But, if the undivided holothurian axohydrocoel is derived, we can predict vestigial structures whose presence reflects a separate axocoel at some time in the past; structures such as pentaradial gonads, or a transitory axial sinus. In addition, the subsidiary functions of the separate axocoel would be subsumed by various organs other than the axohydrocoel alone during the early development or metamorphosis of holothurians. My analysis of the anatomy of *Stichopus californicus* bears out the predictions implicit in the hypothesis that the undivided axohydrocoel is primitive. Of course, it can be argued that the axocoel might have divided off from the axohydrocoel and either been lost or regressed leaving no trace of its existence, but this hypothesis offers no explicit predictions, and cannot be tested.

The importance of the holothurian buccal podia to these considerations is underlined by the general agreement that an ancestral function of the echinoderm water vascular system was to support circumoral feeding tentacles (Bather, 1900; Hyman, 1955; Beklemischev, 1969; Paul and Smith, 1984). Among extant echinoderms, holothurians alone retain this original function. The anterior side of the axohydrocoel gives rise to the coelomic lining of the buccal podia during *Stichopus* development. The buccal podia of holothurians have no direct counterpart among the rest of the echinoderm classes (MacBride, 1914). While the buccal podia are primary podia, and they may bear a photoreceptor (Yamamoto and Yoshida, 1978), they are not at the posterior end of a radial canal as are the primary azygous podia of the remaining classes. These facts led Semon (1888) to hypothesize that the buccal podia of holothurians were homologous with the radial canals of the other classes. This contention caused substantial controversy (Bury, 1895; MacBride, 1914), because it implied that the radial canals of holothurians were not homologous with the radial canals of other echinoderms. However, MacBride (1914) noted that difficulties remained with the homology of the holothurian buccal podia.

I suggest that the separate axocoel in echinoderms could have arisen from the coelomic lining of buccal podia in an ancestral echinoderm with a coelomic configuration resembling modern holothurians. That is, from the part of the undivided axohydrocoel that was specialized for larval attachment. I make this suggestion because the coelomic lining of the buccal podia is derived from the undivided axohydrocoel, because holothurians lack a separate axocoel, because settling juvenile holothurians attach by their buccal podia, and because the buccal podia of holothurians have no obvious homologous structure in the other echinoderm classes. The transition of the coelomic lining of buccal podia from axohydrocoelic attachment organs (which later develop into adult feeding organs), to axocoelic attachment organs (which are lost in the adult), explains both the function and the remarkable structural similarities of the brachiolar attachment organs with tube feet (Barker, 1978), while retaining the homologous tissues of origin.

Conclusions

This description and analysis of metamorphosis in *Stichopus californicus* demonstrates that there are substantial differences between the traditional view of holothurian metamorphosis (Bury, 1895; MacBride, 1914), and the events described here. These differences include characters that appear primitive when compared to the condition in asteroids, ophiuroids, and echinoids, and that may be ancestral among all echinoderms, such as the relative simplicity of the morphogenic movements associated with metamorphosis, the retention of the larval axis of bilaterality and the absence of metamorphic torsions, the absence of an adult rudiment, the presence of coelomic pores, the presence of an internal madreporite, and the lack of a separate axocoel. These differences are not consistent with the generally accepted notion that holothurians are closely related to echinoids.

The idea of shared globose form or shared meridional growth gradients in echinoids and holothurians, an important argument used in support of the assignment of holothurians to the subphylum Echinozoa, reflects only a superficial similarity between these animals. The most significant contradiction to this concept is the absence of axial or visceral torsion in holothurians. This fact explains the radically different orientation of holothurians compared to echinoids. Holothurians and echinoids are just not comparable globose creatures. It should be possible to derive the globose echinoids from the stellate asteroids and ophiuroids, while maintaining the internal organs in their appropriate position (Smith, 1984b). The distinction between a meridional growth gradient in the echinozoa and a radial growth gradient in the stellarozoa may be based on an incorrect comparison of the apical zone of the echinoid aboral surface and the entire aboral surface of asteroids (Fell, 1963). The larger part of the aboral surface of asteroids can be interpreted as interambulacral rather than apical. As to the question of the proposed homology of the echinoid Aristotle's lantern and the holothurian aquapharyngeal bulb, a definitive answer to this conundrum will require detailed functional morphological analysis.

In summary, each point in this study contradicts the hypothesis that holothurians and echinoids shared a common ancestor. The assignment of holothurians to the subphylum Echinozoa should be reassessed. Since this paper argues that holothurians are primitive only in comparison with asteroids, ophiuroids, and echinoids, since this is based only on data derived from metamorphosis, and since Smith (1984a) has reached contrary conclusions, it is prudent to further test the hypothesis that holothurians are primitive echinoderms by constructing a suite of characters, including those described here, and subjecting it to computer assessment for the most parsimonious phylogenetic tree (Smiley, 1986).

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NOREPINEPHRINE AND DOPAMINE CONTENT OF LARVAE AND SPAT OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*

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ABSTRACT

Oyster larvae and spat of varying ages were homogenized and analyzed for catecholamine content using high-performance liquid chromatography with electrochemical detection. Norepinephrine (NE) and dopamine (DA) were found in appreciable quantities, with more DA present than NE. The presence of epinephrine was equivocal. Norepinephrine levels were low in young larvae (0.062 ± 0.035 pg/μg protein), then increased to an intermediate level (0.517 ± 0.031 pg/μg protein) for most of the larval period and finally increased again just before metamorphosis to 1.08 ± 0.09 pg/μg protein. Dopamine levels in the corresponding larval groups did not change significantly, with 11.5 ± 1.9, 8.58 ± 0.98, and 7.47 ± 0.92 pg DA/μg protein, respectively. The levels of NE (1.00 ± 0.04 pg/μg protein) and DA (7.13 ± 0.29 pg/μg protein) present in spat following metamorphosis were not significantly different from the pre-metamorphic values. The presence of NE in oyster larvae at the time of metamorphosis supports the hypothesis of a role for endogenous NE in mediating metamorphosis.

INTRODUCTION

Although norepinephrine (NE) is a major neurotransmitter in the vertebrates, its role(s) in the invertebrates has not been established (Welsh, 1972; Leake and Walker, 1980; Gospe, 1983). Although low levels of NE have been found in most invertebrate phyla, there has been little evidence of a functional role for NE or for the presence of receptors that might mediate such a function. However, Coon *et al.* (1985) have demonstrated that larvae of the Pacific oyster *Crassostrea gigas* (Thunberg), can be induced to metamorphose from a pelagic larval stage to a sessile juvenile stage by a brief (<1 h) exposure to NE or epinephrine (EPI), and subsequent research (see below) suggests catecholamines may be directly involved in natural oyster metamorphosis.

Prior to metamorphosis, *C. gigas* larvae, like many marine invertebrate larvae, are pelagic and must spend some period of growth and development in the plankton. At a certain developmental stage, the larvae become competent to respond to appropriate environmental stimuli, which can cause them to proceed through a series of complex stereotyped behaviors called settlement. If a suitable substrate is encountered during settlement, the larvae will attach to it irreversibly. After attachment, the larvae undergo a morphological and physiological metamorphosis to a sessile juvenile stage. Norepinephrine- and EPI-induced metamorphosis is independent of settlement and attachment; upon exposure to NE or EPI, the larvae sink to the bottom and metamorphose without attaching (Coon *et al.*, 1985).

Coon and Bonar (submitted) have shown that induction of metamorphosis by

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EPI is mediated by receptors pharmacologically identified as alpha$_1$-adrenoceptors, thus suggesting that endogenous NE or EPI may play some role in the metamorphosis of *C. gigas*. Other natural adrenergic agonists, such as dopamine (DA) and octopamine (OCT), for which there is growing evidence for neurotransmitter functions in the molluscs (Gospe, 1983; David and Coulon, 1985), are ineffective inducers of metamorphosis.

We have found no reports in the literature of levels of endogenous catecholamines in molluscan larvae. Norepinephrine has been found in the nervous systems and other tissues of adults of some molluscan species (Juorio and Killick, 1972; Guthrie *et al.,* 1975; Burrell and Stefano, 1983) but except for a report on *Helix* by Osborne (1984), EPI has not been found. Dopamine is considered to be the major catecholamine in the molluscs (Welsh, 1972; Gospe, 1983). This study was undertaken to determine the levels of endogenous catecholamines (NE, EPI, and DA) in *C. gigas* larvae.

MATERIALS AND METHODS

Materials

Oyster larvae were obtained from the Coast Oyster Hatchery (Quilcene, Washington) as previously described (Coon *et al.,* 1985) and homogenized upon arrival. Different size classes of larvae were shipped separately and prior to homogenization were further sorted using Nitex screens of appropriate mesh sizes. The mean size of each group of larvae homogenized was determined by measuring the anterior-posterior length of 25 individuals. All references to larval age are days after fertilization and refer to the age of the larvae when they were shipped from the hatchery.

Post-metamorphic juveniles (called spat) used in this study were produced in our laboratory by allowing batches of larvae to settle and attach onto a clean glass surface for two hours, following which they were carefully scraped free with a razor blade. The spat were then carefully culled and those with damaged shells were discarded. Undamaged spat completed metamorphosis normally and were homogenized at 6 or 24 hours after the beginning of the two hour settlement period.

All chemicals used were the highest grade available from Sigma Chemical Co. (St. Louis, Missouri). The Coomassie Blue dye reagent was obtained from BioRad (Richmond, California). Ultra-pure water (distilled, deionized, and reverse osmosis-purified: DDW) was used for all analyses.

Tissue preparation

Prior to homogenization, larvae and spat were washed twice with iced 0.2 μm filtered seawater. After washing, a quantity of whole larvae or spat was homogenized in a glass tissue grinder in 1.0 ml iced 0.4 N perchloric acid (PCA) containing 4 mM reduced glutathione, 5 mM EGTA, and 7.2 nM dihydroxybenzylamine (DHBA) as an internal standard. Homogenates were stored at −70°C until final processing for analysis. Just prior to analysis, homogenates were thawed and centrifuged at 15,000 × g for 5 minutes and the catecholamines were extracted from the supernatant using alumina (Anton and Sayre, 1962). An 800 μl aliquot of the supernatant was diluted to 3 ml with DDW followed by the addition of 2.0 ml of 1.5 M Tris (pH = 8.6) containing 55 mM Na_2 EDTA. Approximately 50 mg of acid washed alumina was added and each tube was agitated for 15 minutes by gentle inversion on a vertical turntable. The alumina was washed twice with DDW before transferring it to a Microfilter assembly (Bioanalytical Systems, Inc., West Lafayette, Indiana: BAS). The remaining wash was removed by centrifugation and the catecholamines were eluted from the alumina with 100 μl of 0.1 *N* PCA. This extract was injected directly into the HPLC system.

Catecholamine analysis

Catecholamines were separated using a 250 × 4.6 mm I.D. column with 5 μm, C-18 reverse phase packing (Biophase RP-18, BAS). The mobile phase contained 95 m*M* monochloroacetic acid, 1.3 m*M* Na_2EDTA and 0.91 *M* acetonitrile and was adjusted to pH = 3.00–3.05 with NaOH (85 m*M* final concentration). Sodium octyl sulfate (1.2 m*M* final concentration) was added as an ion pairing agent (Knox and Jurand, 1976; Krstolovic, 1982). The flow rate was adjusted to 1.5 ml/min.

Detection of catecholamines was accomplished using an LC-4B amperometric detector with a glassy carbon electrode (BAS) set at an oxidizing potential of 750 mV *versus* a Ag/AgCl reference electrode (Riggin and Kissinger, 1977; Krstolovic, 1982). The detector was set at 0.5 nA/V for all catecholamines except DA, which, because of its high concentration, required a detector setting of 5.0 nA/V; the recorder was set at 1 V full scale.

Catecholamines were identified by comparison of their retention times to those of known standards as a function of increasing concentrations of acetonitrile (0.91–1.7 *M*) and by comparison of their peak heights to known standards as a function of increasing electrode potentials (600–900 mV). Catecholamines were quantified using peak heights compared to a standard curve which was calculated at the beginning of each day. Catecholamine concentrations were expressed as pg of free base/μg protein, with protein content determined by the Coomassie Blue dye binding method (Bradford, 1976) using bovine serum albumin as the standard. Overall recovery for the procedure was about 60% and data were corrected for recovery efficiency. Data were analyzed at the 95% confidence level using analysis of variance and the Newman-Keuls method to test for differences between means (Snedecor and Cochran, 1967).

RESULTS

Figure 1 shows typical chromatographs obtained for a solution of catecholamine standards (A) and for alumina extracts of homogenized competent oyster larvae (B). It can be seen that NE, DA, and the internal standard, DHBA, were well separated from all other peaks and that there were few extraneous peaks. Peak I (probably dihydroxyphenylalanine), peak II (probably epinephrine), and peak III (unknown) in the chromatograph of the larvae could not be adequately identified or quantified so were not included in this report, however these peaks are the subjects of continuing research. Dopamine was the dominant catecholamine in all larval samples. Note the change in scale used for the DA peak in Figure 1(B).

Figure 2 shows the amount of NE present in oyster larvae and metamorphosed spat as a function of shell length. These data are summarized in Table I. Norepinephrine is at a low level (0.062 ± 0.035 pg/μg protein) in young larvae up to about 120 μm in length (5–6 days old). There is an increase in NE content to (0.517 ± 0.031 pg/μg protein) by about 155 μm length (8–9 days old). Norepinephrine remains at about this level for most of the remainder of the larval period until another increase to 1.08 ± 0.09 pg/μg protein occurs at around 300 μm length (18–19 days old). This increase corresponds closely with the onset of metamorphic competence. Figure 2 also shows that during the first 24 h following metamorphosis, the level of NE (1.00 ± 0.04 pg/μg protein) does not change from pre-metamorphic levels.

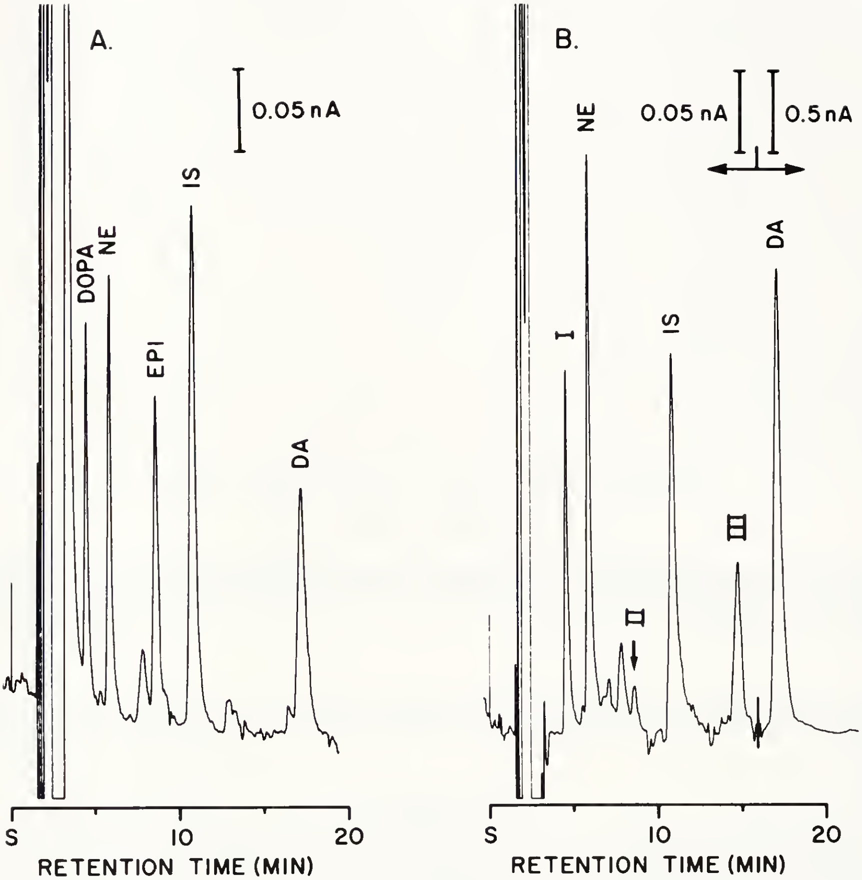

FIGURE 1. Typical chromatographs obtained using HPLC with electrochemical detection as described in the text. (A) Solution of catecholamine standards (DOPA: 230 pg; NE: 340 pg; EPI: 340 pg; IS: 460 pg; DA: 340 pg). (B) Alumina extract of a homogenate of whole, competent oyster larvae. Note the change in scale for the DA peak. DOPA: dihydroxyphenylalanine; NE: norepinephrine; EPI: epinephrine; IS: internal standard, dihydroxybenzylamine; DA: dopamine. Unidentified peaks: I (probably dihydroxyphenylalanine), II (probably epinephrine), III (unknown).

Figure 3 shows the corresponding amounts of DA present in the larvae and spat as a function of larval size. These data are also shown in Table I along with the NE data. It can be seen that there is significantly more DA than NE present in the larvae and spat. There are no statistically significant trends in the DA data although there is a tendency for higher values in smaller larvae; the DA content per μg protein is relatively constant throughout the larval life. The DA levels in small, middle-sized, and large larvae are 11.5 ± 1.9, 8.58 ± 0.98, and 7.47 ± 0.92 pg/μg protein, respectively. Like NE, the DA levels (7.13 ± 0.29 pg/μg protein) do not change during the first 24 h following metamorphosis compared to pre-metamorphic values. Unlike the NE content, there is no increase in the DA content corresponding to the onset of larval competence. The ratio of DA:NE decreases during larval development from a value of about 180 for small larvae to about 17 for middle-sized larvae and finally to about 7 for the largest (competent) larvae and spat. Because there is little change in DA

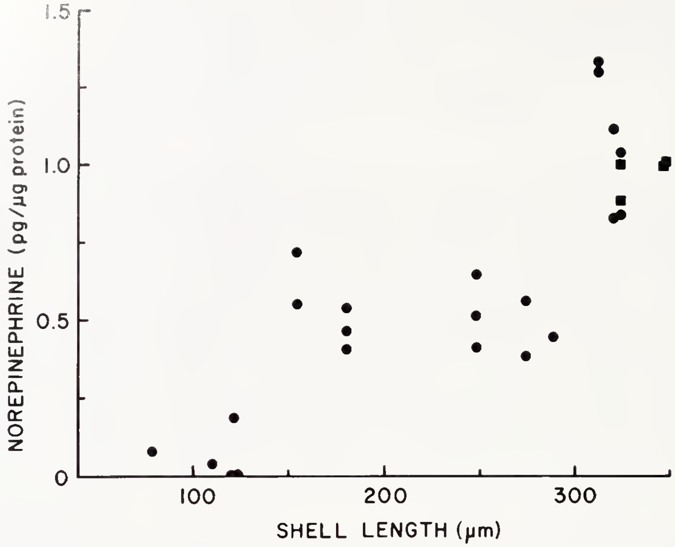

FIGURE 2. Norepinephrine content of oyster larvae and spat as a function of anterior-posterior length. Each point represents a single determination. Circles: larvae; squares: spat 6 and 24 hours after the onset of metamorphosis.

levels during this time, most of the decrease in the DA:NE ratio is due to increases in NE.

DISCUSSION

The present study extends knowledge of the occurrence of catecholamines in molluscs to the larval stages. Norepinephrine and DA are present in appreciable amounts in *C. gigas* while the presence of EPI is still equivocal. However, it is difficult to compare directly the absolute concentrations of catecholamines reported here, which are determined from whole animal extracts (including the proteinaceous shell matrix), with those found in the literature pertaining to adults, which are usually deter-

TABLE I

Catecholamine content of oyster larvae and spat

Shell length (μm)	Norepinephrine content (pg/μg protein)	Dopamine content (pg/μg protein)	DA:NE ratio
Larvae			
[illegible]-122	0.062 ± 0.035 (a)[1]	11.5 ± 1.9 (d)	180
[illegible]-288	0.517 ± 0.031 (b)[2]	8.58 ± 0.98 (d)	17
[illegible]	1.08 ± 0.09 (c)	7.47 ± 0.92 (d)	6.9
Spat			
324-[illegible]	1.00 ± 0.04 (c)	7.13 ± 0.29 (d)	7.1

[1] All values are the mean ± S.E.M.

[2] Means followed by the same letter in parentheses are not significantly different from one another.

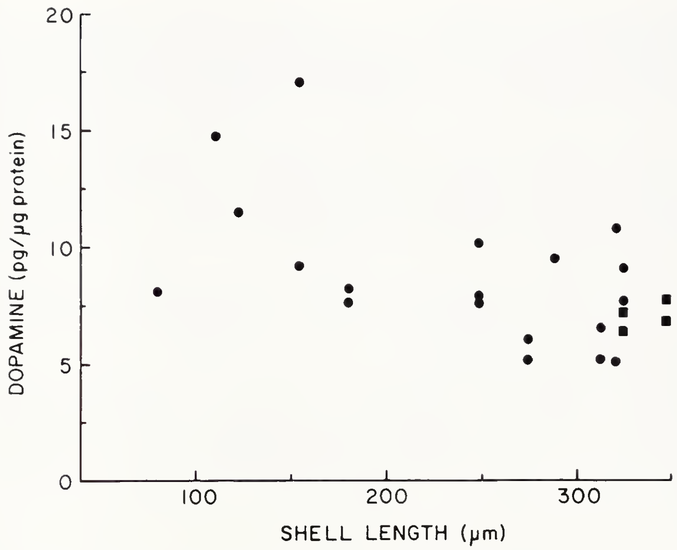

FIGURE 3. Dopamine content of oyster larvae and spat as a function of anterior-posterior length. Each point represents a single determination. Circles: larvae; squares: spat 6 and 24 hours after the onset of metamorphosis.

mined from isolated tissues. Nonetheless, the DA:NE ratio may provide a point of comparison although such comparisons must be made cautiously because of differences in the tissues studied.

As shown in Table I, the DA:NE ratios were 180, 17, and 6.9 for small, middle-sized, and large (competent) larvae, respectively; the ratio for new spat was 7.1, similar to the value for competent larvae. These values agree generally with values from the literature. For example, the DA:NE ratio in molluscan neural tissue was found to range from about 11–13 in *Mytilus* (Burrell and Stefano, 1983) to 20–85 in *Helix* (Guthrie *et al.,* 1975; Osborne, 1984), and with ratios of 17 and 6 for *Helix* kidney and ventricle, respectively (Guthrie *et al.,* 1975). Thus it can be seen that in small oyster larvae the DA:NE ratio is somewhat higher than the literature values, in middle-sized larvae the ratio is similar to literature values and for competent larvae and spat the ratio is somewhat lower.

The presence of NE in competent oyster larvae, along with our previous reports that NE will induce metamorphosis (Coon *et al.,* 1985) and that induction is mediated by putative alpha$_1$-adrenoceptors (Coon and Bonar, submitted), suggests that endogenous NE has a functional role in oyster metamorphosis. This is believed to be the first evidence for a specific functional role for NE in the molluscs. Glaizner (1968) has reported an identified neuron in the snail *Helix* which is stimulated specifically by NE, however the function of this neuron is unknown.

The apparent increase in NE levels near the onset of metamorphic competency is intriguing, especially in light of its presumed involvement in metamorphosis. Since little is known about the physiological changes that accompany competency, the role of NE in this phenomenon is difficult to evaluate. Perhaps noradrenergic neurons are forming, or existing neurons are accumulating NE, either in preparation for involvement in the processes of settlement and/or metamorphosis, or for post-metamorphic functions. The observation that NE levels do not change following metamorphosis implies that NE has some additional function(s) besides its suspected role in metamorphosis.

Although peak II (see Fig. 1B) was present in nearly all samples tested, its identity as EPI could not be unequivocally verified. Its magnitude was found to vary greatly, independent of larval size and several sample preparation procedures. Although peak II always coeluted with authentic EPI as a function of changing concentrations of acetonitrile in the mobile phase, in some samples its peak height did not correlate well with authentic EPI as a function of the detector potential, indicating the presence of a contaminating compound in some samples. We have not yet been able to resolve this problem, but in light of the fact that EPI is the most effective catecholaminergic inducer of metamorphosis (Coon *et al.,* 1985; Coon and Bonar, submitted) and that there is only one other report of EPI in a mollusc (Osborne, 1984), we are continuing to pursue the identification of peak II.

The process of oyster metamorphosis is complex, involving many different tissues and including histolysis, histogenesis, and the accompanying biochemical and physiological changes (Cole, 1938; Hickman and Gruffydd, 1971). The control mechanisms coordinating these diverse changes are unknown (Burke, 1983; Chia and Rice, 1978; Hadfield, 1978) and the role of NE in this process is likewise unclear. Norepinephrine could be acting as a neurotransmitter within the central nervous system to release a hormone that would act on the peripheral target tissues (centralized receptor theory), analogous to the control of neurosecretory release of a growth hormone by dopamine in the snail *Lymnaea stagnalis* (Stoof *et al.,* 1984). Alternatively, NE could be acting directly on the target tissues (peripheral receptor theory) either as a neurotransmitter or as a hormone. An action on target tissues is suggested in another mollusc, the nudibranch *Phestilla sibogae,* where NE at high concentrations induces low levels of partial metamorphosis in which a specific larval tissue, the velum, is lost but subsequent morphological changes do not occur (Hadfield, 1984). The present data give no indication of the location of either NE or DA within the larvae because whole animal homogenates were used. Further research on the localization of NE and the alpha$_1$-adrenoceptors is required to more fully evaluate the actual role of NE in oyster metamorphosis.

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PARTHENOGENESIS IN *CARCINONEMERTES* SPP. (NEMERTEA: HOPLONEMERTEA)

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ABSTRACT

When juveniles of *Carcinonemertes* spp. are removed from male crabs and raised in the laboratory, a 50:50 sex ratio is obtained. Isolated females produce egg strings typical of *Carcinonemertes,* except that the embryos are haploid, with about 13 chromosomes per cell. Larvae develop at least to hatching, and some do hatch. Nearly all of the 146 females so raised produced egg strings. Larvae of all females raised in isolation were haploid while larvae of females put with males were diploid. Females from both U. S. west coast species, *Carcinonemertes epialti* and *C. errans,* and from three hosts, *Cancer magister, C. antennarius,* and *Hemigrapsus oregonensis,* produced these haploid larvae by parthenogenesis.

INTRODUCTION

Nemerteans in the genus *Carcinonemertes* live on crabs. Two species of *Carcinonemertes* have been recorded from the U. S. west coast: members of *C. errans* occur on *Cancer magister,* the commercially important dungeness crab (Wickham, 1978), and it is thought that members of *C. epialti* inhabit the other U. S. west coast crabs that harbor worms (Wickham and Kuris, 1985). Most crab species along this coast harbor worms, but the number of species of *Carcinonemertes* that actually occur along this coast is still unknown.

Carcinonemertes juveniles occur on both male and female crabs (Kuris, 1978; Roe, 1979; Wickham, 1980). Male crabs apparently harbor only juvenile worms, since resident worms move from male to female crabs during crab mating (Kuris, 1978; Wickham *et al.,* 1984). Juveniles apparently do not feed, but are maintained by absorbing dissolved organic materials, at least part of which are leaked through the arthrodial membranes of their host's skeleton (Crowe *et al.,* 1982). After a female crab ovulates, her resident worms migrate to the egg clutch (Humes, 1942; Kuris, 1978; Roe, 1979; Wickham, 1980). During the crab's brooding season the worms feed on the developing crab larvae, grow, and reproduce. Each female worm makes several egg strings (Humes, 1942; Wickham, 1979, 1980; Roe, 1984).

Worms can reach enormous numbers on individual hosts, and because they feed on developing zoeae they have been implicated in the decline of the dungeness crab fishery along the U. S. West Coast (Wickham, 1979). Since their feeding biology apparently affects host populations more than other aspects of their lives, most recent studies have focused on these worms as "egg predators" (Roe, 1984; Wickham, 1979; Wickham and Kuris, 1985).

Members of *Carcinonemertes* spp. are definitely predators during their trophic phase on brooding crabs, but most of their life history characteristics are parasitic.

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Parasites have evolved several adaptations to increase reproductive output, including asexual reproduction, increased egg production, hermaphroditism, and parthenogenesis. The present study reports the first evidence for parthenogenesis in *Carcinonemertes.*

MATERIALS AND METHODS

Worms were raised in isolation (experimental worms) or in groups (controls) in 5-cm diameter plastic disposable petri dishes, either in the constant temperature chamber at California State University, Stanislaus, or on running seawater tables at the Bodega Marine Laboratory. Temperature was maintained close to normal seawater temperature, *i.e.,* 12–14°C. Since it is not known how many species comprise the *Carcinonemertes epialti* group, worms were separated by host. To raise worms, juveniles were removed from male crabs. One juvenile worm was placed with a clump of crab eggs in a petri dish. Similar containers held several worms together as controls. Eggs of the crab *Hemigrapsus oregonensis* were used as food throughout these experiments because brooding females of *H. oregonensis* are collected easily throughout the year. It was previously determined that all worms tested will feed on eggs of *H. oregonensis* (Roe, 1984). Containers were checked every 2–4 days as worms grew. Crab eggs and seawater were changed on a haphazard basis, whenever the eggs or water became fouled or a worm needed more food.

When female worms began laying egg strings in the culture dishes, the egg strings were observed using compound or stereomicroscopes to see if eggs were dividing. In addition, some egg strings of both experimental and control worms were stained with aceto-orcein so that chromosomes in the eggs could be counted. To stain with aceto-orcein, an egg string was removed from the culture dish and put into another 5-cm petri dish containing 3 parts absolute ethanol:1 part glacial acetic acid for at least one hour. The egg string was then placed on a microscope slide with a drop of aceto-orcein (2% aceto-orcein dissolved in 75% acetic acid). A cover slip was quickly placed over the egg string and pressure was applied to compress the eggs and to make some eggs break out of the egg case. These temporary slides were ringed with fingernail polish. The next day the coverslip was removed and a drop of CMCP-9AF (Masters Chemical Co., Inc.), mounting media was placed on the egg string and the cover slip was replaced, to make a permanent slide. All methods to make permanent slides without waiting at least 24 hours resulted in poor chromosome staining.

Stained egg strings were observed under oil at 1000× with a compound microscope and chromosomes were counted.

RESULTS

Juvenile worms placed with crab eggs for food grew to maturity in about 3 weeks (males) to 30 days (females) under the laboratory conditions of these experiments. As seen in Table I, the individuals that grew obtained approximately a 50:50 sex ratio. Females developed slower than males and are about twice the size of males at maturity. These problems can easily account for the slightly fewer females than males in two of the three experimental populations. There is no reason to think that one sex predominated among those worms which, for one reason or another, never grew.

In addition, there was approximately a 50:50 sex ratio (47 females, 43 males) of *C. epialti* on *Hemigrapsus oregonensis* when all worms were counted from 7 ovigerous crabs collected from nature.

Females of both *Carcinonemertes errans* and *C. epialti* produce egg strings even

TABLE I

Numbers of worms of Carcinonemertes *spp. raised for observations of parthenogenesis*

Species		Number raised			
Worm	Host	Total	Females	Males	Other*
Carcinonemertes errans	*Cancer magister*	320	117	137	66
Carcinonemertes epialti	*Hemigrapsus oregonensis*	75	17	21	37
Carcinonemertes epialti	*Cancer antennarius*	40	12	12	16
		435	146	170	119

* No development, died, etc.

when they are isolated from all other members of their species. Females started making egg strings about 30 days after experiments began. Both experimental and control females laid several egg strings, usually with one to one-and-one-half day intervals between each string. Eggs are 65–75 μm in diameter. An average of 152 (±108 S.D.) eggs occurred in 14 strings randomly chosen from 71 strings of experimental *C. errans.*

Most egg strings were laid before the eggs had completed meiosis; one and then two polar bodies could be seen during the first one to one-and-one-half hours after an egg string was produced. When egg strings were stained with aceto-orcein during polar body formation, 12 or 13 chromosomes usually could be counted, both in the polar body and remaining in the cell, in both experimental and control worms of both species (Table II, Fig. 1a). From these data it was determined that the haploid number of chromosomes for both *C. errans* and *C. epialti* is probably 13. Polar bodies can be seen through the 2-cell stage in developing worm embryos. However, by this time the chromosomes have started to fragment, clump together, etc.; this can account for cells with both very low and high numbers seen in Table II. In addition, chromosomes often seem to lie on top of one another, and it is sometimes difficult to separate them; this could account for most of the low numbers in both Tables II and III.

Mitotic chromosomes were easiest to count when cells in the egg strings were dividing to the 2-cell stage. After the 4-cell stage, cells became small enough that it was usually difficult to count chromosomes. Early mitotic divisions in embryos of controls of both *C. errans* and *C. epialti* showed the diploid number of 24–26 chromosomes (Table III, Fig. 1b). However, embryos from experimental (isolated) females of both *C. errans* and *C. epialti* showed the haploid number of chromosomes even during mitosis to 2, 4, and later cell stages (Table III, Fig. 1c). A total of 434 cells in approximately 103 egg strings from 35 different experimental females of both species showed that virtually all embryos produced by experimental females were haploid (Table III).

Eggs in egg strings of experimental females developed, at least to the hatching point. When larvae of *Carcinonemertes* hatch they are multicellular and are fully ciliated. Larvae from experimental females appear to be normal except that they seem to have a harder time actually hatching from the egg case than do larvae produced from fertilized eggs. Some larvae from isolated females did hatch, and these larvae lived several days post-hatching, similar to larvae that develop from fertilized eggs of control females.

Although the production of 1N embryos by isolated females of *Carcinonemertes*

Table II

Number of cells counted from egg strings of Carcinonemertes *spp. showing the number of meiotic chromosomes listed*

Worm category	Number of chromosomes per cell <10	10	11	12	13	14	15
Experimental[1]							
Carcinonemertes errans (20 egg strings from 13 females)	5	10	26	41	28	11	
Carcinonemertes epialti from *Hemigrapsus oregonensis* (14 egg strings from 9 females)	1	4	7	19	20	1	
Control[2]							
Carcinonemertes errans (6 egg strings)	1	5	8	8	7	2	
Carcinonemertes epialti (9 egg strings)	4	5	8	11	13	2	2

[1] Experimental worms are those raised in isolation.
[2] Control worms are those raised in groups.

spp. has only been observed in laboratory conditions, it does not appear to be a laboratory artifact. Egg strings were produced by 126 of the 146 experimental females. The 20 females that did not produce eggs included worms that died or were removed from observation before they started reproducing, and a few individuals that did not

Table III

Number of cells counted from egg strings of Carcinonemertes *spp. showing the number of mitotic chromosomes listed*[1]

Worm group	Number of chromosomes per cell 9	10	11	12	13	14	15–19	20	21	22	23	24	25	26	27
Experimental[2]															
Carcinonemertes errans (71 slides from 24 females)	9	30	68	92	101	3					1?	2 + 1?			
Carcinonemertes epialti from *Hemigrapsus oregonensis* (32 slides from 11 females)	2	5	21	43	50	6									
Control[3]															
Carcinonemertes errans (18 slides)							10	8	5	11	10	7	4	4	1
Carcinonemertes epialti (22 slides)			1	2	3		22	7	2	12	6	6	4	2	

[1] Chromosomes of 5 cells/egg string usually counted; 1 egg string per slide in most cases; ½ total number of chromosomes/cell recorded for anaphase and early telophase.
[2] Experimental worms are those raised in isolation.
[3] Control worms are those raised in groups.

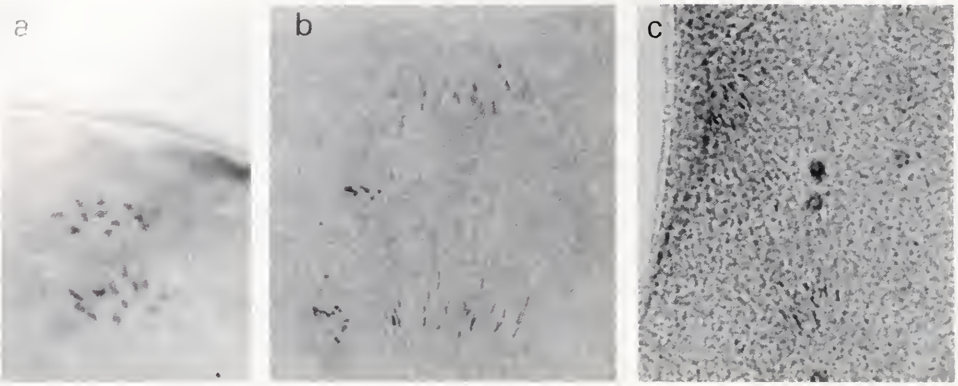

FIGURE 1. a. Polar body formation in egg of control *Carcinonemertes epialti.* Chromosomes nearest edge of the cell are those of the polar body. Some cells showed 12, other cells showed 13 chromosomes during meiotic divisions. Magnification: 1500×. b. Chromosomes in mitotic anaphase during division from one to two cell stage in embryo of control *Carcinonemertes errans.* The small dark spots to the left of the chromosomes are remains of chromosomes in the two polar bodies. Magnification: 1000×. c. Chromosomes in mitotic anaphase during division from two to four cell stage in haploid embryo of experimental *Carcinonemertes errans.* Chromosome number is one-half that of Figure 1 b. Magnification: 1000×. Controls are worms raised in groups. Experimental worms are worms raised in isolation.

begin to feed until many days after the experiments started and therefore were very slow to develop. Some of these females never developed to the point of laying eggs. In addition, of the 440 egg strings of experimental animals observed either with a compound or stereomicroscope to determine if the eggs were dividing, at least 430 strings had dividing, developing eggs. Finally, after two experimental females had made egg strings with haploid embryos, males were added to their culture dishes. Of the eight egg strings checked for chromosome numbers that were made after males were introduced, two egg strings had haploid embryos and six had diploid embryos.

DISCUSSION

These studies show that females of *Carcinonemertes* can produce offspring by parthenogenesis when necessary. That larvae produced by isolated females are indeed produced by parthenogenesis is supported by several lines of evidence. The primary evidence is that these larvae are haploid. In addition, there is no question that these females are indeed females and not self-fertilizing hermaphrodites. Both in cultures and in nature one sees a 50:50 sex ratio. Males and females are easily distinguished (Roe, 1984). Animals were isolated as juveniles, before any gonadal development had occurred. In addition, only juveniles from male crabs were used; all evidence indicates that only juvenile worms inhabit male crabs (Wickham *et al.,* 1984). All lines of evidence indicate that these experimental females produced unfertilized eggs capable of development.

Although the phenomenon of parthenogenetic egg production by *Carcinonemertes* has only been observed in the laboratory, it is thought that parthenogenesis might be a normal part of the life history of these worms. Nearly all 146 females raised in these experiments did produce eggs, and eggs in virtually all 440 strings observed were seen to be dividing and developing. In addition, *Carcinonemertes* often occurs in very low numbers on many crab species (Wickham, in press), and in such circumstances a female worm would be unlikely to find a mate on her particular host. Parthenogenesis could be a decided adaptive advantage in such situations of low numbers and little mobility.

It remains to be seen whether parthenogenesis in *Carcinonemertes* has ecological significance. A small percentage of parthenogenetically produced larvae do hatch, and appear to behave normally. However, no one has been successful to date in getting any larvae of *Carcinonemertes* spp., normal or parthenogenetic, to settle or grow into juveniles in laboratory conditions. So it is not yet known if parthenogenetic larvae can grow to maturity or if they would be able to reproduce.

A total of 5 apparently diploid larvae were seen in approximately 15,000 cells (430 egg strings × average 152 cells per string) (Table III plus one observation seen before chromosomes were systematically counted from all egg strings). In some of these five cases, the cells appeared in thicker areas on slides, and it could not be determined for certain that cells were not lying on top of one another and chromosomes actually belonged to two cells. However, in most cases the cells appeared to be clearly diploid. In these situations the diploid cells were in embryos that were dividing to 4 cell stage or were already at 4 cell stage. It appears that perhaps in a low percentage of parthenogenetic larvae, the diploid condition is achieved. The mechanism by which diploidy is achieved in this particular situation is not known, but in other parthenogenetic systems, in which meiosis has occurred, it has been found that one or the other polar bodies can fuse with a cell nucleus (Whitfield and Evans, 1983) or the two nuclei of the first mitotic division can fuse (Bell, 1982). It is reasonable to assume that any diploid larvae in the egg strings of parthenogenetic *Carcinonemertes* would have at least an equal chance of hatching as haploid larvae and perhaps a better chance. If these larvae then settled onto a crab and matured, they could reproduce in the normal manner for *Carcinonemertes* and the effort of females to reproduce even in isolation would be an advantage to the population. An apparently similar situation is obtained in all-female populations of the rat schistosome, *Schistosoma douthitti* (summarized in Whitfield and Evans, 1983). In these populations, offspring are produced by parthenogenesis, and most (over 95%) are haploid males. A low frequency of 2N males does occur among the parthenogenetically generated embryos, probably from fusion of the haploid nuclei in early stages of developing embryos. The 1N offspring seem to have low fitness and viability, but the parthenogenetically produced 2N males appear to possess a fitness similar to normally produced offspring.

Although it is by no means limited to parasites, parthenogenesis is a common adaptation of parasites to increase their reproductive output (Price, 1980). Such an adaptation, in animals that show several other traits of parasites as well, characterizes the parasitic nature of *Carcinonemertes.* This idea is supported by negative evidence from the free living nemerteans *Cerebratulus* and *Lineus. Cerebratulus* eggs were refractive in response to reagents which readily induce artificial parthenogenesis in some animals (Morse, 1912). Most eggs only developed polar bodies in these experiments, although a few treatments resulted in eggs dividing to the 2 or 4 cell stage; nothing induced development past the early morula stage (Morse, 1912). In studies on *C. lacteus,* Freeman (1978) found that eggs do not develop even the first polar body unless they are fertilized. Unfertilized eggs of *Lineus ruber* can start cleavage, but the cleavage is irregular. Mitoses are multipolar and development is abortive (Langlet, 1972, summarized in Bierne, 1983).

Finally, these studies can be a useful tool in determining the actual number of species of *Carcinonemertes* that actually occur along the U. S. west coast. If males and females from different hosts are raised together, and if only 1N offspring are produced, then we can suspect the worms are different species. However, if worms from different hosts mate and produce 2N offspring, then we should expect that they are the same species or very closely related, and that environmental conditions determine the morphological differences we see in worms from different hosts.

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IMMUNOCYTOCHEMICAL STUDY OF THE RED PIGMENT CONCENTRATING MATERIAL IN THE EYESTALK OF THE PRAWN *PALAEMON SERRATUS* PENNANT USING RABBIT ANTISERA AGAINST THE INSECT ADIPOKINETIC HORMONE

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ABSTRACT

Using antisera produced against different parts of the insect adipokinetic hormone (AKH), it was possible to detect adipokinetic hormone-reactive peptides in the eyestalk of the prawn *Palaemon serratus.* Immunopositive staining was obtained in some neurosecretory cells of the medulla externa X organ (MEX), of the medulla terminalis X organ 2 (MTGX-2), in the lower part of the sinus gland, and in the nerve joining the medulla terminalis X organs to this neurohemal gland. The biological activity of synthetic AKH and RPCH (red pigment concentrating hormone) was tested on the movement of the red pigment in the chromatophores of the prawn and compared to the activity of extracts derived from immunoreactive tissue regions. The inhibiting effect of the AKH antibodies on the biological activity was ascertained by immuno-adsorption experiments. The results are discussed in relation to the molecular resemblance of AKH and RPCH. It is postulated that RPCH-material in the eyestalk of *Palaemon serratus* can be identified by using antisera against AKH-peptide. However, more than one type of immunoreactive RPCH/AKH-like peptide appears to be present in different groups of neurosecretory cells.

INTRODUCTION

Color changes in Crustacea are produced by the movement of pigments inside the different types of chromatophores. This pigment migration is regulated by a set of neurohormones, the chromatophorotropins. Among them the red pigment concentrating hormone (RPCH), extensively investigated in numerous species of prawns, supplies a model for the control mechanism of color adaptation in Crustacea.

Perkins (1928) and Koller (1928) provided the first experimental data pointing to the hormonal influence of the eyestalk on the crustacean color changes. Since then innumerable studies have given evidence for the physiological role of the eyestalk chromatophorotropins, particularly of RPCH (see reviews in Carlisle and Knowles,

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According to personal communication comparable results have been obtained by S. Mangerich, H. Dircksen, and R. Keller in their manuscript "Immunocytochemical identification of putative red pigment concentrating hormone-containing structures in the central nervous system of decapod crustaceans" (in press).

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1959; Kleinholz, 1961; Fingerman, 1963; Bellon-Humbert, 1970; Noël, 1981). Present identification and characterization of RPCH have allowed the production of synthetic analogs. These preparations have fostered the study of structure-activity relationships (Otsu, 1965; Fernlund, 1968, 1974; Fernlund and Josefsson, 1968, 1972; Fingerman, 1973; Skorkowski, 1973; Josefsson, 1975; Carlsen *et al.,* 1976; Jaffe *et al.,* 1982). Furthermore, the purification of pigment concentrating factors from the corpora cardiaca, neurohemal centers in insects, led to the isolation and characterization of the insect adipokinetic hormones, AKH (I) and AKH (II) (Goldsworthy *et al.,* 1972; Stone *et al.,* 1976). These two neuropeptides are close structural analogs to the crustacean RPCH (Mordue and Stone, 1978; Josefsson, 1983). Recently, one of the authors (H.S.) and his colleagues identified AKH-like material in the locust and other insects using C-terminal and N-terminal specific antisera to AKH (I) (Schooneveld *et al.,* 1983, 1985a, b, 1986).

In spite of the detailed knowledge of the RPCH molecule and its physiological function, information about the cells synthetizing the RPCH material in the eyestalk of Crustacea was not available until now. Consequently the pattern of cellular activities such as synthesis, processing, storage and release is unknown. The suggestion that a neurosecretory cell group in the MEX organ of the eyestalk may be a production center for RPCH active material was provided some years ago by two of us (C.B. and F.v.H) using extirpation- and injection-procedures (Bellon-Humbert *et al.,* 1981).

The objective of the present study was to collect more information about RPCH and its presence in the neurosecretory system of the eyestalk from the prawn *Palaemon serratus* using C-terminal and N-terminal specific antisera to AKH (I).

MATERIALS AND METHODS

Collection and maintenance of prawns

Adult prawns, *Palaemon serratus* Pennant, were collected in summer and in winter in the Bay of Concarneau (France). They were maintained in running seawater at the normal season temperatures (17°C in summer—11°C in winter), with the natural photoperiod. The animals were fed daily with synthetic food pellets and fresh mussels. For each experiment, prawns were selected according to their size (55–60 mm) and molting cycle (intermolt C-stage).

Immunocytochemical experiments

Between 9:00 and 10:00 a.m., eyestalks were removed from prawns in C stage of their molting cycle. They were fixed in Bouin-Hollande fluid containing 10% of a saturated aqueous solution of sublimate, then dehydrated, cleared according to the conventional histological procedure, and embedded in Histomed (melting point 58°C). Serial sections 7 μm thick (longitudinal and transversal) were deparaffinized, washed in Lugol and a hyposulphite solution, and rinsed in distilled water before equilibration in 0.05 *M* Tris-HCl buffered saline, pH 7.6. Then the sections were immunocytochemically stained by the peroxydase-antiperoxydase (PAP) method (Sternberger, 1979), using 4-Cl-1-naphthol as a marker for the peroxydase activity. The best results were obtained with the following incubation procedure: (1) Normal goat serum (dilution 1:5): 10 min incubation; (2) Anti-AKH-serum (code 241 or 433) (dilution 1:750–1:1000): incubation for 24 to 48 h at 4°C; wash; (3) Goat anti-rabbit IgG (H + L) serum (dilution 1:20): 20 min; wash; (4) PAP complex (dilution 1:50): 30 min; wash; (5) Substrate incubation (100 mg 4-Cl-1-naphthol in 0.05 *M* Tris-HCL, pH 7.6, containing 0.005% H_2O_2): 7 min; wash in distilled water; (6) Mounting in water medium.

The primary antisera were produced in rabbits against a complex of synthetic (Tyr^1) AKH and thyroglobulin (code 241/3-6-82: Schooneveld *et al.*, 1983) and against AKH (1–4) also conjugated to thyroglobulin (code 433: Schooneveld *et al.*, 1985b). The former antiserum is referred to as a C-terminal specific, the latter as an N-terminal specific antiserum (Schooneveld *et al.*, 1986). The second antiserum [GAR IgG (H+L)] and the PAP complex were obtained from Nordic (Tilburg, The Netherlands). The specificity of the immunocytochemical staining method was tested by successively substituting each of the incubation reagents in the normal procedure for buffer and by using the anti-AKH serum preadsorbed with 10 nmol synthetic AKH or synthetic RPCH/ml. The controls were carried out on sections adjacent to those stained with the complete immunoenzyme cytochemical procedure. Sometimes azan-staining was used to identify the immunoreactive cells and structures on the sections.

Physiological experiments

To obtain more information about the biological activity of the red pigment concentrating (RPC) material in the different eyestalk structures of the prawns, injection experiments were carried out. The erythrophorotropic activity of the following tissue extracts was tested: sinus gland; medulla terminalis X-organs (as the $MTGX_1$ and $MTGX_2$ are not easily removed from the circumscribed cells, the medulla terminalis was extracted as a whole); medulla externa X-organ (MEX); organ of Bellonci; medulla interna, externa and lamina ganglionaris material; whole eyestalk. Synthetic AKH and synthetic RPCH (Peninsula Labs) were also tested. In addition the biological activity was compared in sample series non treated and pre-treated with the rabbit anti-AKH-serum code 241.

Preparation of the tissue extracts, dilution series of samples, and injection procedure. The aforementioned eyestalk structures were dissected from the eyestalks of large prawns in molting stage C and collected in icy-cold saline (distilled water + filtered seawater 1:1). After homogenization in a microglass grinder, the extracts were centrifuged (3000 × *g*; 20 min; 4°C) and the supernatants were collected and lyophilized. For each extract a dilution series of 1, 0.1, 0.01, and 0.001 equivalent/10 μl saline was prepared. The two synthetic peptides were tested in a dilution series of 1 pmol, 100 fmol, 10 fmol, and 1 fmol/10 μl saline. All samples were injected into the abdominal region between the muscles and the cuticle from eyestalkless intermolt prawns, using a thin needle microsyringe. Eyestalk ablation was carried out 24 h before injection, so that pigments were fully expanded (index 5).

Preincubation of the samples with antisera. To study the effect of the immunochemical reaction of the anti-AKH sera on the biological activity of selected samples, 1 μl undiluted antiserum (code 241) was added to 100 μl of the dilution series of synthetic AKH, RPCH and two tissue samples (MEX organ extract and sinus gland extract). These solutions were gently mixed and incubated for 1 h at 37°C then 24 h at 4°C. Afterwards, they were centrifuged (20,000 × *g*; 5 min; 4°C), the supernatants collected and used for injection experiments in comparison to the non-treated samples.

Chromatophore index estimation. The two types of erythrophores, the large ones drawing a specific pattern of bands and the small ones, scattered in their intervals, were examined. The small erythrophores were the most reactive. In the data presented here, only their activities were retained. Measurements were recorded in Panouse's scale (1946) using 6 stages characterizing the pigment dispersion grade (0: full concentration, punctiform chromatophore; 1: irregular shaped chromatophore; 2: stellate chromatophore with a few large chromorhizae; 3: sea urchin-like aspect with bifurcated chromorhizae; 4: highly bifurcated and fine chromorhizae, chromatophores still distinct; 5: full dispersion, adjacent chromatophores with confused chromorhizae). The measurements were always recorded in the dorsal area of the first abdominal segment on pools of 10 animals for each sample and at intervals of 10 min up to 60 min. All the physiological experiments were performed between 10 and 12 a.m., in natural daylight conditions and on a white background.

RESULTS

Immunocytochemical observations

Immunopositive reactions with both anti-AKH sera were detected in some neurosecretory cells of the medulla externa and medulla terminalis, in the sinus gland, as well as in the nervous tract, starting in the medulla terminalis X organ 2 and running up to the neurohemal region (Fig. 1). While some immunoreactive cells were localized at the base of the medulla terminalis, most of them were found in two neurosecretory cell groups: the medulla externa X organ or MEX and the medulla terminalis X organ 2 or MTGX-2.

In the MEX, on the dorsal side of the eyestalk, 1 to 4 cells were strongly stained with the anti-AKH serum code 241 and faintly with the anti-AKH serum code 433. These cells are characterized by a large size (diameter about 30–32 μm), a round and large nucleus (diameter about 13–15 μm), and a rough granular cytoplasm. They are localized inside the organ and are covered by small neurons (Fig. 2).

In the MTGX-2, on the ventral side of the eyestalk, a variable immunostaining with sera 241 and 433 was observed in 1 to 5 cells. These cells are located between the proximal part of the organ of Bellonci and the cells synthetizing the hyperglycemic hormone or CHH (Van Herp *et al.*, 1984). The diameter of the cells reacting with the two antisera ranges from 20 to 30 μm. But sometimes, 1 to 2 larger cells (diameter ⩾30 μm) in the center of the MTGX-2 were also immunoreactive (Fig. 3).

The immunostaining of the sinus gland was obvious and striking: only the inner and lower part of the gland (proximal part) contains axon terminals stained with both anti-AKH sera (Figs. 4, 5). These terminals belong to neurons in the MTGX-2 area as it was possible to visualize partial portions of the nerve joining the MTGX-2 and the sinus gland (Figs. 4, 6). In the medulla terminalis, in the medulla externa, and in the lamina ganglionaris, a positive reaction to AKH antibodies of the C terminal serum was observed in grouped nerve fibers (Fig. 1). The perikarya of these nerves could not be identified with certainty.

After successively substituting each of the incubation reagents with buffer or after incubation with anti-AKH serum adsorbed with AKH or RPCH, no elements were stained. These control data are summarized in Table I. Because the cells at the basis of the medulla terminalis (Fig. 1) remain positive after incubation with normal rabbit serum, their immunoreaction was interpreted as non specific.

Physiological effects of tissue extracts and peptides

In an attempt to relate the presence of immunoreactive material to well characterized physiological parameters, the RPCH activity of the different immunoreactive

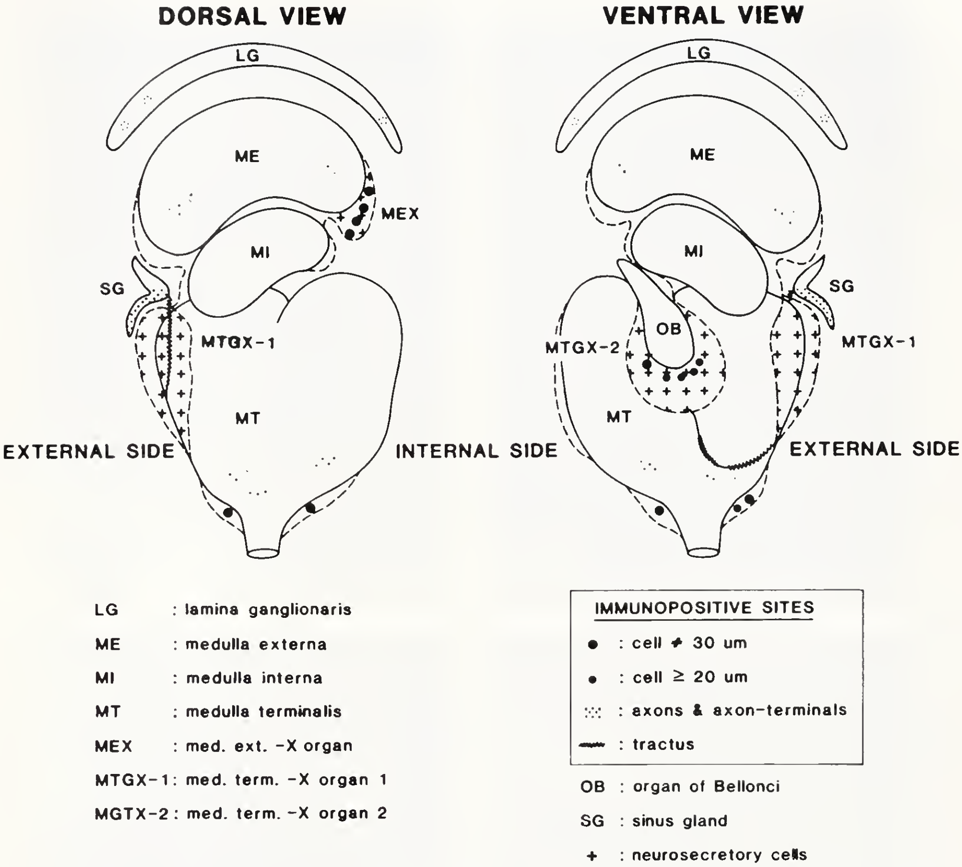
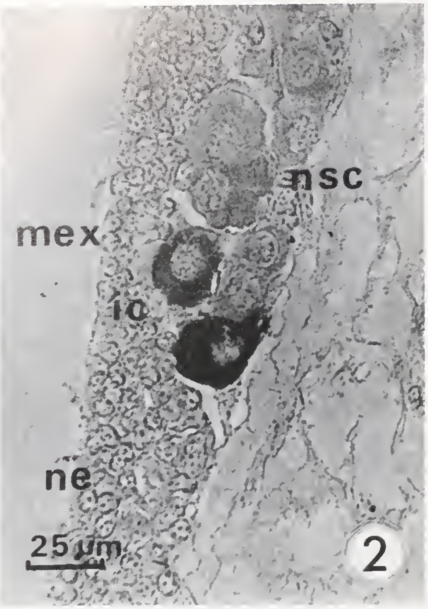
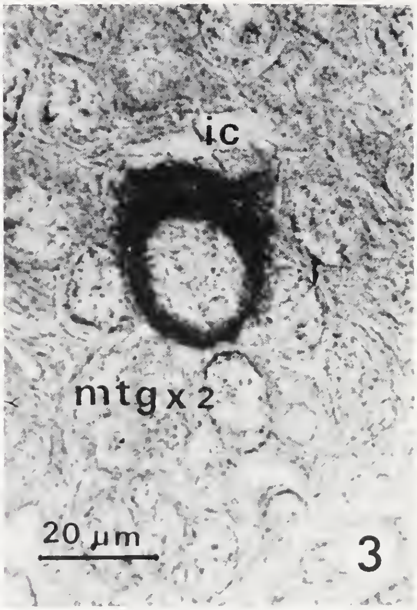

DORSAL VIEW
VENTRAL VIEW
LG
ME
MI
MEX
SG
MTGX-1
MTGX-2
OB
MT
EXTERNAL SIDE
INTERNAL SIDE
EXTERNAL SIDE
LG : lamina ganglionaris
ME : medulla externa
MI : medulla interna
MT : medulla terminalis
MEX : med. ext. -X organ
MTGX-1 : med. term. -X organ 1
MGTX-2 : med. term. -X organ 2
IMMUNOPOSITIVE SITES
● : cell ≥ 30 um
• : cell ≥ 20 um
∴ : axons & axon-terminals
〰 : tractus
OB : organ of Bellonci
SG : sinus gland
+ : neurosecretory cells

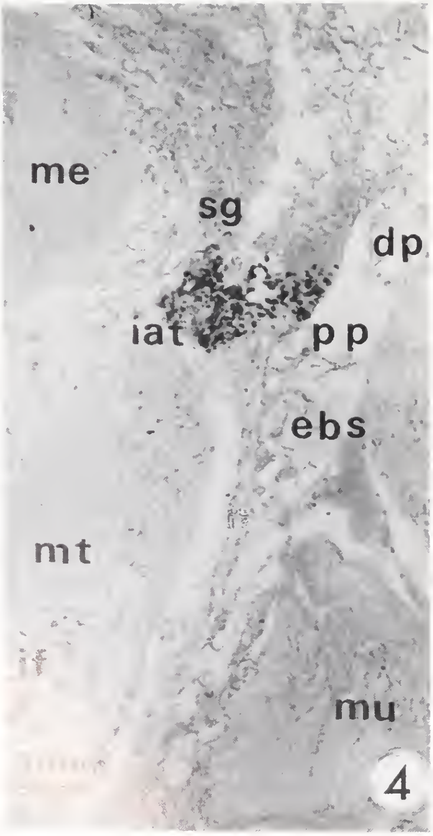
FIGURE 1. General organization of the eyestalk of *Palaemon serratus* with the indication of the immunopositive sites for anti-AKH sera.

structures of the eyestalk was investigated. Extracts of total eyestalks or of selected organs were prepared (see Materials and Methods) and one organ equivalent or less was injected into individual eyestalkless prawns. In these prawns the red pigment was maximally dispersed. All bioassay results were compared to the chromatophorotropic activity of the total eyestalk (Figs. 7A, B). MEX extracts were more active than MT extracts (MTGX-1 and 2) and comparable to the activity of the whole medullae (LG, ME, MI). Sinus gland extracts were very potent and approached the activity of total eyestalk extracts. A strong concentration of the red pigment was observed after the injection of extracts of the organ of Bellonci, in spite of the lack of reaction with the anti-AKH sera.

The MEX and the sinus gland were selected for answering the question if the physiological response of extracts showed a dose dependency. As Figures 8A and B show, this is clearly the case. The intensity of the response is compared to that of pure RPCH and AKH, tested simultaneously (Figs. 8C, D). It appeared that the biological activity of 1 sinus gland was comparable to that of 1 pmol RPCH and superior to


nsc
mex
ic
ne
25 µm
2


ic
mtgx2
20 µm
3


me
sg
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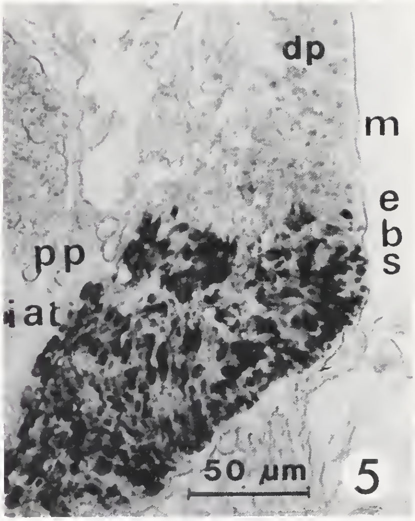
mu
4


dp
m
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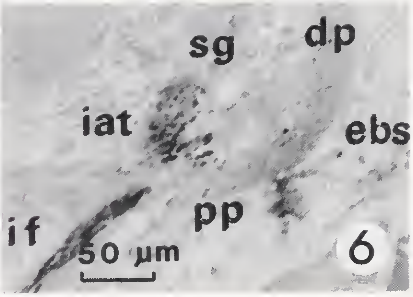
50 µm
5


sg
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6

TABLE I

Immunostaining in the eyestalk of the prawn Palaemon serratus *after incubation with anti-AKH sera (code 241 and code 433) and control tests*

			Immunoreactive sites			
Antiserum	Dilution	Incubation	SG	MEX	MTGX-2	Tractus
Code 241	1/750	24 h	+++	(+)	+	+
Code 241	1/750	48 h	+++	+++	++	++
Code 433	1/1000	24 h	++	−	+	+
Code 433	1/1000	48 h	++	(+)	+	+
NRS		24 h	−	−	−	−
AKH/anti-AKH (code 241)	1/750	24 h	(+)	−	(+)	−
RPCH/anti-AKH (code 241)	1/750	24 h	−	−	−	−
RPCH/anti-AKH (code 433)	1/1000	24 h	(+)	−	−	−

+++: very strong staining; ++: intermediate staining; +: weak staining; (+): doubtful staining; −: no staining; NRS: normal rabbit serum.

that of 1 pmol AKH while the activity of 1 MEX organ corresponded approximately to 10 fmol RPCH or 100 fmol AKH.

The chromatophorotropic activity of organs and synthetic AKH and RPCH after preincubation with the anti-AKH serum (code 241) was tested to obtain additional evidence that the immunoreactive structures are in fact responsible for the RPCH effects of tissue homogenates. Figures 9A and B show that immunoprecipitation of AKH-like material did not decrease the activity of the sinus gland extracts, when the concentration of 1 and 0.1 structure equivalent/10 μl saline was used. MEX extracts lost their biological activity almost completely if concentration comprised between 1 or 0.1 MEX equivalent/10 μl saline were pretreated with antiserum (Figs. 10A, B). The synthetic hormones RPCH and AKH did not lose their activity by this procedure. No significant effect on the RPCH activity was detected with preincubated RPCH, whatever the concentration was; a slightly perceptible effect was only noticed for the preincubated AKH, when the concentration was 100 fmol (results not shown).

FIGURE 2. Detail of the neurosecretory cells (nsc) of the MEX-organ (mex), showing the situation of the immunopositive cells (ic) bordered by neurons (ne).

FIGURE 3. Large immunopositive cell inside the MTGX-2 organ (mtgx2). Note the very strong reaction with the anti-AKH serum.

FIGURE 4. Longitudinal section of a right eyestalk, showing the sinus gland (sg) with its two regions: proximal (pp) and distal (dp) parts. Immunopositive axon terminals (iat) are located in the proximal part. Note also the immunopositive fibres (if) of the nerve joining the sinus gland to the MTGX-2 through the medulla terminalis (ebs: external blood sinus; mu: muscle).

FIGURE 5. Detail of a longitudinal section of the sinus gland. Note the immunopositive axon terminals in the proximal part of the gland in contrast to the immunonegative axon terminals of the distal part (m: outer membrane of the gland).

FIGURE 6. View of the sinus gland with immunopositive fibers and axon terminals of the nerve originated from MTGX-2.

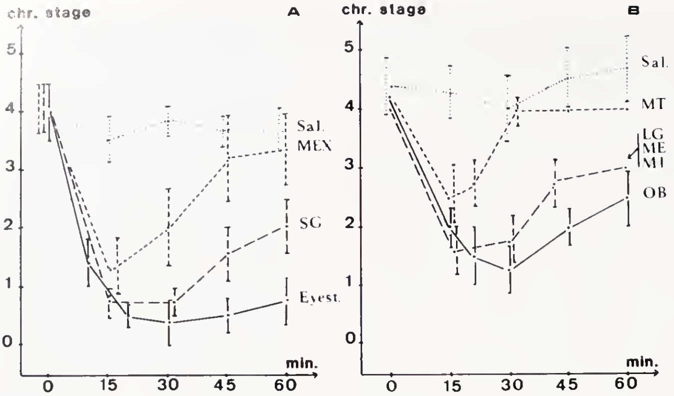

FIGURE 7A, B. Effect of extracts of different eyestalk structures on the red pigment concentration in the small erythrophores of the prawn (Sal: saline; Eyest.: eyestalk; LG, ME, MI: lamina ganglionaris + medullae externa and interna; MEX: X-organ of the medulla externa; MT: medulla terminalis without OB; OB: organ of Bellonci; SG: sinus gland).

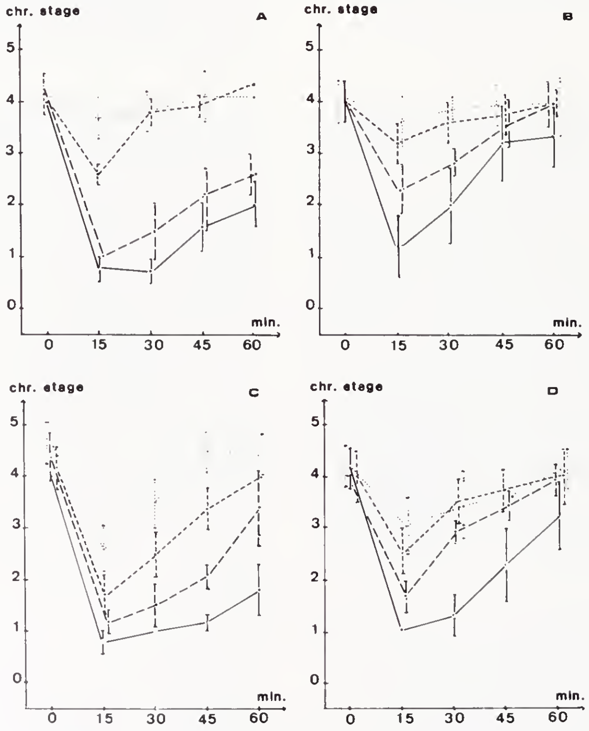

FIGURE 8. Red pigment concentrating activity of the sinus gland (A) and MEX (B) extracts, compared to the activity of the synthetic peptides RPCH (C) and AKH (D). A and B (——: 1 structure equivalent/10 μl; — — —: 0.1 structure equivalent/10 μl; -----: 0.01 structure equivalent/10 μl; · · · ·: 0.001 structure equivalent/10 μl) compared respectively to C and D (——: 1 pmol hormone/10 μl; – – –: 100 fmol hormone/10 μl; - - -: 10 fmol/10 μl; · · · ·: 1 fmol/10 μl).

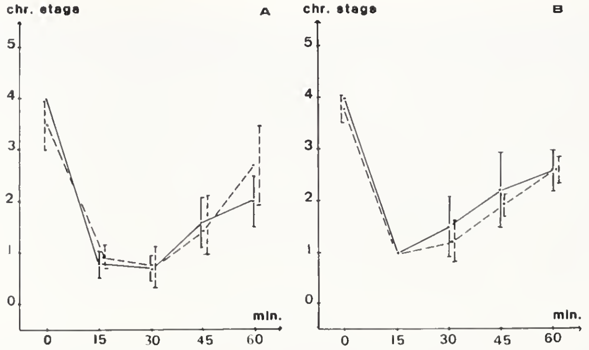

FIGURE 9. Red pigment concentrating activity of the sinus gland extracts, pre-incubated with the anti-AKH serum code 241 (——: untreated extracts; – – –: pre-incubated extracts; A: 1 SG equivalent/10 μl; B: 0.1 SG equivalent/10 μl).

DISCUSSION

The detection of RPCH/AKH-like material in the eyestalk of the prawn *Palaemon serratus* was based on the chemical relationship which exists between the crustacean RPCH and the insect hormone AKH. The molecular structure of the AKH (Stone *et al.*, 1976) closely resembles that of the RPCH (Fernlund, 1974) and these two substances "reproduce each other's biological effects when cross-tested on members of the two arthropod groups" (Mordue and Stone, 1976). These properties suggested the use of two different anti-AKH sera to reveal the sites producing and storing RPCH/AKH-like material in the eyestalk of the prawn and to compare the biological activities of tissue extracts with those of synthetic AKH and RPCH.

The antisera used were raised in rabbits against (Tyr^1) - AKH (Tyr-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2) coupled through its N-terminal to thyroglobulin (code 241) and against the AKH (1–4) peptide (pGlu-Leu-Asn-Phe) also coupled to thyroglobulin through its C-terminal (code 433) (Schooneveld *et al.*, 1983, 1985b,

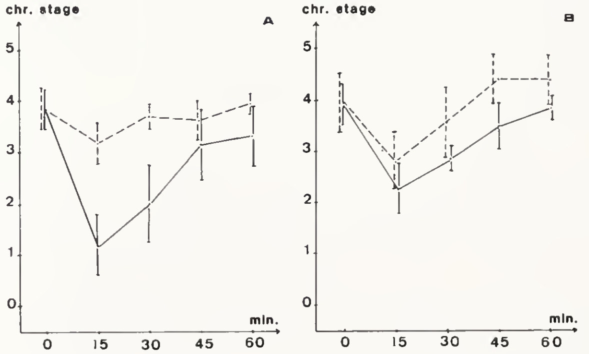

FIGURE 10. Red pigment concentrating activity of the MEX extracts, pre-incubated with the anti-AKH serum code 241 (——: untreated extracts; – – –: pre-incubated extracts; A: 1 MEX equivalent/10 μl; B: 0.1 MEX equivalent/10 μl).

1986). With both antisera we could visualize the same morphological structures: some neurosecretory cells in the MEX and MTGX-2 organs, one part of the sinus gland, and a tract. The homologous staining in the mentioned structures implies that neuropeptidergic material containing the common amino acid sequence (-Leu-Asn-Phe-) reacts with the antisera. As the RPCH molecule in prawns has the same amino acid sequence in the same position (pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2), it is possible that the material identified by the antisera belongs to the PCH family. The fact that the immunocytochemical reaction of the anti-AKH sera is mostly inhibited with synthetic AKH as well as with synthetic RPCH, gives additional support to this hypothesis. However, at this time it cannot be excluded that other molecules with similar epitopes react positively with the antisera.

The synthesis of AKH-like material in the cells of the MEX was expected: we already mentioned the progressive dispersion of the red pigment when this organ was removed and the concentrating effect induced by the MEX-extracts (Bellon *et al.,* 1981). We were able to distinguish in this organ four cell types according to their morphological characteristics and staining properties (Van Herp *et al.,* 1977). The immunoreactive cells can be classified as α or β cells because of their size, nucleus shape, and location.

AKH-like material was also detected in some neurosecretory cells of the medulla terminalis, chiefly in the MTGX-2 organ. This organ is considered to be the main source of neurohormones in the eyestalk, since the axons originating from the neurosecretory cells form the only important nerve running to the sinus gland. These cells occur in small numbers and have variable size and secretion content. Apparently, these cells can be classified as ϵ and π cells, according to Van Herp *et al.* (1977). The differences in stainability may be related to the cellular rhythms in synthetic activity (diurnal and molting cycles), but also to the background adaptation and illumination conditions. We noticed that the strongest immunocytochemical reaction was observed in some of the largest cells of the MTGX-2 organ.

The immunoreaction in the sinus gland and in the tract with the AKH antisera implies that the immunoreactive material is carried by the nerve tract from the neurosecretory cells of the medulla terminalis and mainly stored in the lower part of the gland. The restricted immunoreaction in the sinus gland points to the unequal distribution of axon terminals, containing the neurosecretory granules. In fact, on the ultrastructural level, there was no difference in the distribution of the five neurosecretory granule types previously described (Strolenberg *et al.,* 1977). The electron density and the granule size therefore cannot be used to differentiate neurosecretory products from each other. Immunocytochemical methods may help further differentiate granule populations in the electron microscope.

The lack of immunological tracing of a nerve tract originating from the MEX organ to the sinus gland and the presence of immunoreactive fibers in the lamina ganglionaris set the problem of the release of AKH-like material produced in the MEX neurosecretory cells and of the origin of the fibers observed in the upper ganglia. It is possible that this statement reflects a functional difference between the peptidergic cells in the MEX and MTGX-2 organs and that a different mode of release is correlated respectively to neuromodulator and neurohormonal functions. Moreover, during embryogenesis, the medulla externa and medulla terminalis develop differently (Elofsson, 1969; Bellon-Humbert, 1985) and a double chiasma, crossing the axons, affects the medulla externa.

Special attention has been paid to the question of whether the material demonstrated immunocytochemically was responsible for the physiological effects on chromatophore activation of tissue extracts containing such immunoreactive material. If the sinus gland and MEX extracts give clear and expected results, the medulla terminalis was found less active, when the organ of Bellonci was removed. The red pigment concentration induced by extracts of this last organ was carefully controlled. However, this effect remains difficult to explain. Immunocytochemically no AKH-like material is detectable. Analogs to vasopressin and neurophysin (Van Herp and Bellon-Humbert, 1982) and to 5-HT (unpub. results), have no effect on the red pigment concentration. Although we cannot exclude that a few small cells, containing AKH-like material and which are closely in contact with the proximal part of the organ of Bellonci, are injected together with this organ. This can also explain the relatively small effect of the medulla terminalis extracts.

We attempted also to eliminate the physiological factor from the tissue extracts by immunoprecipitation with the anti-AKH serum (code 241) in watery incubation medium. The experiments were performed with the tissues containing appreciable amounts of biologically active material, *i.e.,* the sinus gland and the MEX of the eyestalk. Using fixed amounts of antiserum, the doses of sinus gland extract assayed did not lose their RPC effects. The dose of antibody used (1 μl) presumably was too low to inactivate larger quantities of active factor. This factor could well be authentic RPCH.

The results with the MEX were promising. We showed that the largest dose of MEX extract was fully inactivated by the antiserum. Why the low dosage retains some of its activity remains to be investigated. A possible explanation is that low concentrations of immune complexes are less easily removed from the incubation medium by our centrifugation procedure. From the data obtained in our experiments using synthetic peptides, we deduce that the biological active sites, consisting in the tryptophan residue of the RPCH peptide (Carlsen *et al.,* 1979; Josefsson, 1983) are possibly not always blocked by the anti-AKH serum. Our results with synthetic RPCH and AKH show that no loss of activity can be observed if solutions of peptides are pretreated with anti-AKH serum (code 241). This would be in agreement with earlier observations with immunodiffusion experiments indicating that no precipitation of AKH-IgG complexes can be visualized in gels (Schooneveld, unpub. result). In recent immunoprecipitation tests using goat anti-rabbit IgG serum to precipitate the entire complex, we observed that the primary complex (synthetic RPCH anti-AKH) is only precipitable by addition of a second antibody as GAR (Van Herp, unpub. result). If the first complex indeed remains in solution, the failure to inactivate the biological activity of the peptide perhaps can be attributed to the reversibility of the formation of this antigen-antibody complex. The peptide may then have the option to associate either with the antibody or with the peptide receptors in tissues. The latter bond may be the stronger of the two, leaving the antibodies (partially) unoccupied.

The fact that MEX extracts could be inactivated by preincubation with antiserum implies that the immunoreactive substance in this organ might be related to a physiologically active factor. But whether this factor is RPCH or some other related molecule remains to be investigated by more elaborate immunoprecipitation experiments and chemical analysis. It should be born in mind that insects appear to contain a wide variety of AKH-related peptides in endocrine centers as well as in neurons of the central nervous system. Using the antisera also used here, immunoreactive substances proved to differ from each other in certain staining properties (Schooneveld *et al.,* 1986). Moreover, different peptides related to AKH, including RPCH, were able to mimic AKH in the locust bioassay (Siegert *et al.,* 1985). It cannot be excluded the [illegible] that a similar family of related peptides also occurs in our prawn. One indication of a peptide diversity is the observation that the cells in the MEX hardly stain with antiserum 433, whereas a strong staining is obtained with antiserum 241 (Table I).

The partial adsorption of MEX extracts with the antiserum (due to the precipitation of the immunocomplex sometimes visualized after centrifugation) is another indication that the AKH-like material in the MEX-organ is different from that in the sinus gland. In this last structure, the erythrophoretic activity can be related to a small neuropeptide, as shown by the comparable physiological effects of the synthetic hormone, while the MEX-material can be a large polypeptide, precipitable by the antiserum.

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THE CELLULAR STRUCTURE OF LYMPHOMYELOID TISSUES IN *CHIMAERA MONSTROSA* (PISCES, HOLOCEPHALI)

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ABSTRACT

The ultrastructure of the main lymphomyeloid organs of the holocephalan fish, *Chimaera monstrosa,* is presented.

The thymus is well developed even in adults. It is densely packed with small and large lymphocytes, the former predominating. The ultrastructure of *C. monstrosa*'s lymphocytes is similar to that of mammalian ones. The lymphocytes show intimate connections with large epithelial reticulocytes, which infiltrate the cytoplasm of the lymphocytes with long processes.

The lymphomyeloid tissues around the orbit are mainly granulocytopoietic. Two types of granulocytes occur: eosinophilic and heterophilic. The latter is found in excess. The granulocytes are ultrastructurally similar to those of elasmobranchs such as *Etmopterus spinax* and have similar characteristic granules. In addition to granulocytes in various stages, lymphocytes, plasma cells, blast cells, and solitary macrophages are scattered throughout the tissue.

The suprapalatal region also has a similar supply and variety of cells, but most heterophilic granulocytes have modified granules.

In the spleen, erythrocytes in different developmental stages are common. This confirms earlier studies which suggested that the spleen functions primarily as an erythrocytopoietic organ.

INTRODUCTION

In the mid-nineteenth century, Leydig (1851, 1857) described lymphoid tissue in the orbital and in a suprapalatal region of a holocephalan fish, *Chimaera monstrosa.* The presence of lymphomyeloid tissues in the head of holocephalans has been noticed by a few other authors. Kolmer (1923) investigated such tissue in the suprapalatal region and ascribed it a bone marrow-like function. Holmgren (1942) briefly described the distribution of lymphoid tissues in the craniums of *Chimaera* and *Hydrolagus.* Fänge and Sundell (1969) later studied the histology of these tissues in *Chimaera.* They showed that the cranial lymphomyeloid tissues of *Chimaera* contain aggregates of granulocytes, lymphocytes, and developmental stages of these cell types. Stahl (1967) reported the occurrence of a similar lymphomyeloid tissue in the shoulder region and noticed a close association of this tissue with blood sinuses. A histological study of the spleen of *Chimaera* was performed by Scatizzi (1932) who reported that the less developed white portions were lymphocytopoietic and that the red parts produced granulocytes and erythrocytes.

Previous studies used light microscopy only. The present investigation shows the ultrastructure of the lymphomyeloid organs of *Chimaera monstrosa.* The organs are discussed according to the following designations: the orbital tissue including the pre-

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orbital and suprapalatal tissue, the thymus, and the spleen. Probably lymphomyeloid functions are not confined to these organs but also occur in, for example, intestinal regions. However, from a morphological point of view the tissues mentioned are the most predominant. As far as we know, no previous ultrastructural studies have been performed on these tissues.

MATERIALS AND METHODS

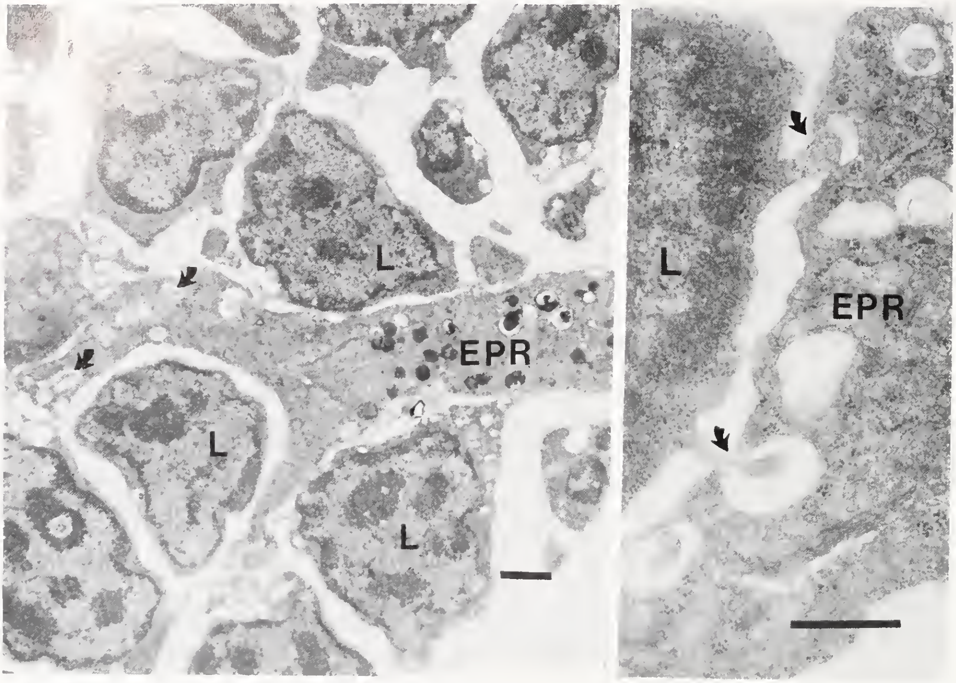
About 20 specimens of the holocephalan *Chimaera monstrosa* were caught by trawling at depths between 100 and 500 m in the region of Skagerrak to the north of Jutland, Denmark, and in a region about 10 miles to the west of Lysekil on the Swedish west coast. Samples were fixed on board the ship 10–20 minutes after the fish were captured.

In preparation for electron microscopy, samples were prefixed in 3% glutaraldehyde for 1–4 days. The glutaraldehyde was dissolved in a mixture of seawater and veronal acetate buffer, 2:1, and adjusted to neutral pH with NaOH. For some samples the glutaraldehyde solution was supplied with sucrose to a final concentration of 10% and a trace of $CaCl_2$ was added (*cf.*, Bell *et al.*, 1969). After washing 3–5 times in the mixture used as solvent for glutaraldehyde, the samples were postfixed in 1% OsO_4 in 0.1 *M* veronal acetate buffer pH 7.4. Dehydration in increasing concentrations of ethanol was followed by embedding in Epon 812. Sections were cut with an LKB ultrotome and contrasted with uranyl acetate and lead citrate. The sections were observed and photographed in an Hitachi HS-8 or in a Zeiss 109 electron microscope.

RESULTS

The cells of the various lymphomyeloid tissues of *Chimaera monstrosa* have light microscopic characteristics which enable investigators to compare them with corresponding cells of other vertebrates (Fänge and Sundell, 1969). The same relationship exists with regard to the ultrastructure of these cells. Most evident similarities exist when comparing the lymphomyeloid cells of *Chimaera* with those of the elasmobranchs, *Ginglymostoma cirratum* and *Etmopterus spinax,* described previously (Fänge and Mattisson, 1981; Mattisson and Fänge, 1982).

Thymus. The anatomy of the thymus has been described previously by Hammar (1912) and by Fänge and Sundell (1969). It consists of medulla and cortex and undergoes only slight age involution. It lies in the roof of the pharynx close to the dorsal parts of the gill arches, posterior to the suprapalatal lymphomyeloid tissue and well separated from it by connective tissue. The thymus is densely packed with lymphocytes with their characteristic, small cytoplasmic volume. Together with the lymphocytes there are large irregular cells of type macrophages as well as interdigitating cells or epithelial reticulocytes (Fig. 1, 2). Lymphocytes can be small or large, however small lymphcytes predominate. Intermediates between these two lymphocytes exist and might be medium-sized lymphocytes or large ones sectioned in a non-equatorial plane. The lymphocyte nuclei possess a large amount of heterochromatin mostly located adjacent to the nuclear envelope. The nuclei are mostly non-lobed, roughly spherical, and supplied with distinct nucleoli. A thin rim of cytoplasm covers the nuclei and the small lymphocytes have a very small proportion of cytoplasm. The cytoplasm is moderately electron-dense and abundant in free ribosomes. The large lymphocytes often have pseudopodia and peripheral electron-lucent vesicles which indicate an endocytotic activity. Both types of lymphocytes often come into contact with epithelial reticulocytes (Fig. 1). The interdigitating processes of these cells sur-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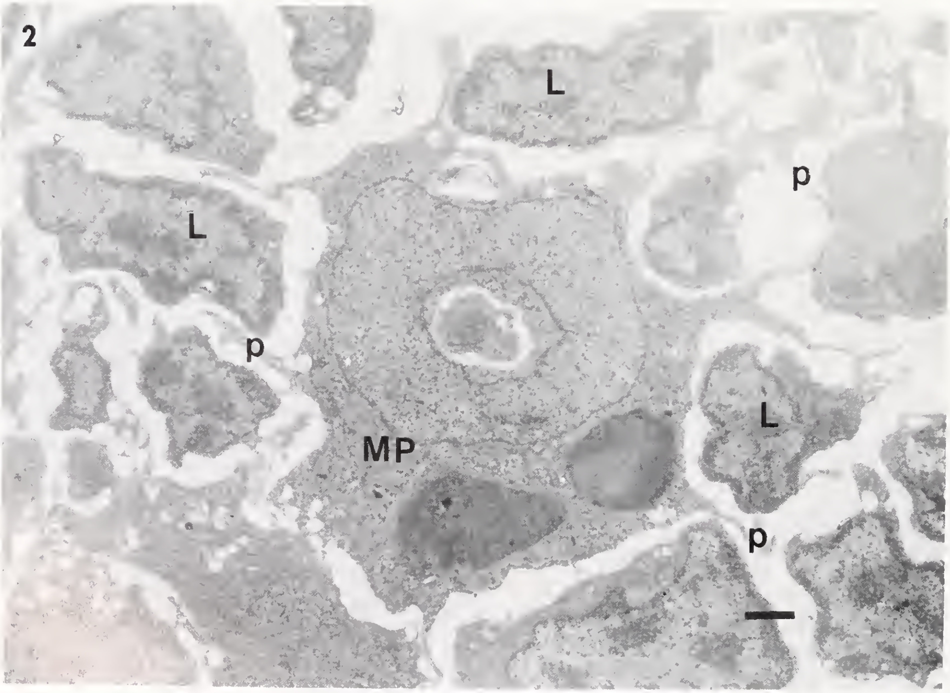

round a number of lymphocytes and the latter often pierce the peripheries of the epithelial reticulocytes. The extensive surface connection between the two types of cells does not cause ultrastructural changes except for an increase of pseudopodia in the border areas. The cytoplasm of the epithelial reticulocytes is supplied with bodies having a strongly electron-dense interior. Within a section, the epithelial reticulocytes might exceed lengths of 100 μm. Macrophages containing various kinds of inclusions are scattered in the thymus (Fig. 2).

The orbital and the preorbital tissues. A whitish, soft tissue surrounds the eyes and, to a large extent, fills up the preorbital (ethmoidal) canal of the cranium. This tissue is mainly granulocytopoietic; the granulocytes constitute about 80% of all the cells. The granulocytes occur in two principal forms. Their light microscopic appearance and their staining characteristics suggest that they are eosinophilic and heterophilic (neutrophilic). The ultrastructure of the granules supports this classification. Among the granulocytes, the heterophils constitute more than $\frac{4}{5}$ of the total number. The two forms show a heterogeneous distribution.

The eosinophils (Fig. 3) have a roughly spherical form and are up to 15 μm in diameter. The eccentric nucleus is non-segmented, mainly euchromatic. In the cytoplasm, there are layers of rough endoplasmic reticulum, which often occur peripherally and run parallel to the cell membrane. The granules structurally resemble those of the eosinophils of an elasmobranch, *Etmopterus spinax* (Mattisson and Fänge, 1982), *i.e.,* they form strongly and homogeneously electron-dense spheres up to 1.5 μm in diameter and are bordered by a membrane (Fig. 4). As shown for *Etmopterus,* the eosinophilic granules often have a halo of different thickness. The halo is most evident close to the well-developed Golgi zone. Sometimes the halo is separated from the dense interior by a zone with an intermediate electron density (Fig. 3), but usually there is an abrupt border between the dense center and the completely electron-lucent periphery (Fig. 4). Some rod-like granules also occur.

The heterophils are somewhat smaller than the eosinophils, up to 13 μm in diameter, and have more irregular shapes. The nuclei are slender and lobed, often horseshoe shaped, with marginal heterochromatin (Fig. 5). The nucleus form is often highly irregular (Fig. 6). A small number of mitochondria are scattered within the cytoplasm. Electron-dense dots indicating calcium deposits often occur within the mitochondria. A well-developed Golgi apparatus probably supplies the cells with the granules which are scattered throughout the cytoplasm. The granules show a great diversity within the same cell (Fig. 5). Some appear like rods but most often they are ovoid with lengths between 0.5 and 1 μm. Several granules contain a well demarcated spheric "empty" region which may occupy almost the entire granule. Similar "empty" vesicles are common in the peripheries of the cells. The dense zones of the granules often show great variations in appearance. Sometimes the dense zones are

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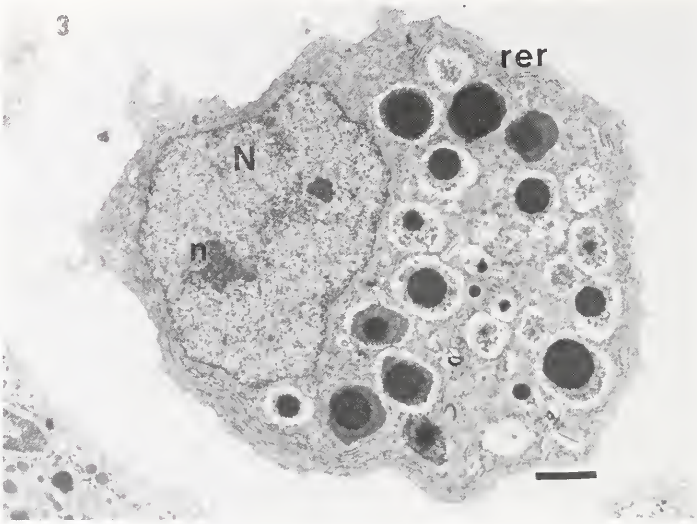
FIGURE 1. Portion of thymus. An epithelial reticulocyte (EPR) makes close contacts with several lymphocytes (L). At the border regions, projections and inwards bents (arrows) facilitate a probable interchange. Areas with strongly electron-dense inclusions are common in the cytoplasm of the epithelial reticulocyte. Fixation: 3% glutaraldehyde in a neutral solution of $\frac{2}{3}$ seawater and $\frac{1}{3}$ veronal acetate, supplied with sucrose and Ca^{++}. Postfixation in 1% OsO_4 in veronal acetate buffer. Bars: 1 μm.

FIGURE 2. A macrophage (MP) and some lymphocytes (L) in the thymus. Two cells are engulfed and others are surrounded by long projections (p) from the macrophage. The center of the macrophage appears pierced by a portion of a cell. Fixation as in Figure 1 but without sucrose and Ca^{++} added. Bar: 1 μm.



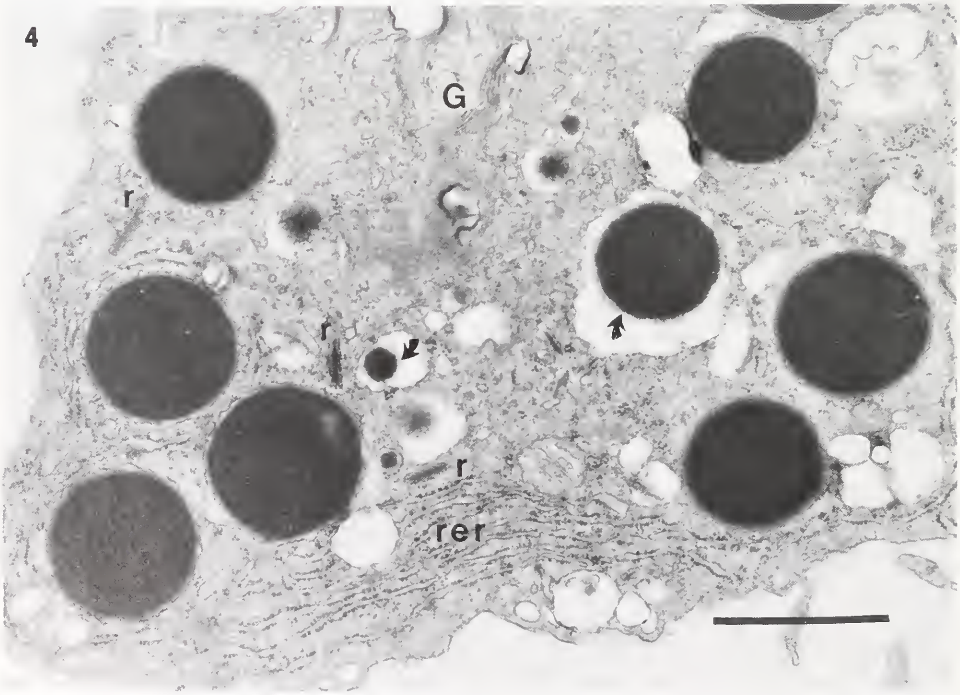


FIGURE 3. Eosinophil from the orbital region. A peripheral nucleus (N) with a nucleolus (n). A peripheral rough endoplasmic reticulum (rer). The spherical cell has spherical granules with the density increasing towards the centers, sometimes a layered density. Fixation as in Figure 2. Bar: 1 μm.

FIGURE 4. Portion of an eosinophil from the orbital organ. Strictly spherical and strongly electron-dense granules supplied with membranes. Close to the Golgi complex (G), there are small introductory stages of granules. An abrupt transition in density occurs in some granules (arrows). In the cytoplasm a peripheral rough endoplasmic reticulum (rer) and some rod-like inclusions (r). Fixation as in Figure 1. Bar: 1 μm.

filamentous or granular but they may also have an interior marked dense area, within which the "empty" region occurs (Fig. 7).

Intermingled with the granulocytes there are aggregations of small lymphocytes with a similar appearance to those of the thymus, *i.e.,* large dense nuclei and thin margins of cytoplasm. Larger cells which appear like large lymphocytes, monocytes, or promyelocytes are few. They contain 2–6 dense granules in each section. The granules are round, about 0.3 μm in diameter, and have a thin marked halo and slightly undulated outlines (Fig. 8). Their ultrastructure agrees with that of granules described as azuruphilic (*cf.,* Bessis, 1973).

In certain areas of the orbital tissue there are small groups of plasma cells. The plasma cells constitute less than 5% of all the cells. They are up to 15 μm in length and often show margins that are undulated (Fig. 9a). Their cytoplasm is filled with the characteristic rough endoplasmic reticulum forming vesicular structures with a faintly electron-dense interior (Fig. 9b). The comprehensive rough endoplasmic reticulum together with the well-developed Golgi zone and the large nucleolus are all indications of an extensive protein production of these cells. Some lysosome-like bodies as well as mitochondria intermingle with the endoplasmic reticulum. In accordance with what was shown for the plasma cells of *Etmopterus spinax* (Mattisson and Fänge, 1982) the heterochromatin of *Chimaera* plasma cells does not show an arrangement like the spokes of a wheel, said to be characteristic of vertebrate plasma cells (*cf.,* Bessis, 1973).

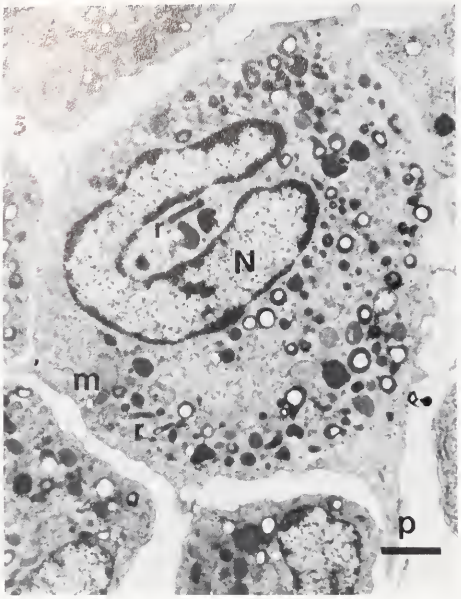
Together with the fully developed cells are blast cells, most of them probably granuloblasts. Solitary macrophages also occur.

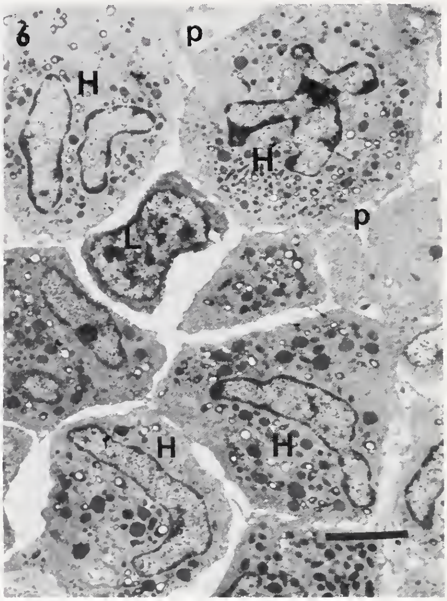
The suprapalatal lymphomyeloid tissue. This tissue lies in the roof of the mouth in the median line at the level of the anterior gills. It is situated in a triangular depression in the cartilage forming the base of the cranium.

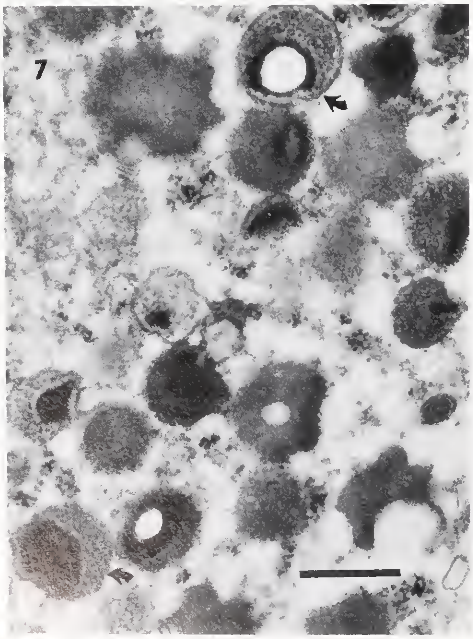
As in the orbital tissue, granulocytes are the predominant cell type within the suprapalatal tissue and constitute at least ⅘ of the cells. A small portion of them are eosinophils. The organelles of these cells have an ultrastructure in accordance with that of the eosinophils of the orbital tissue. Most of the granulated cells are heterophilic with their cytoplasm filled with smaller granules. However, most of the heterophils differ markedly from those of the orbital tissue. The granules are spherical and rather small, only about 0.5 μm in diameter (Fig. 10). They are often faintly electron-dense with a non-homogeneous and variform interior and they lack the central "empty" region characteristic of heterophils from the orbital organ. The nuclei often show a low electron density. But like the heterophils of the orbital tissue, the nuclei are lobed and mostly exhibit a horseshoe-like shape.

Within some areas of the suprapalatal tissue aggregations of small lymphocytes are interspersed among the granulocytes. There are also solitary clusters of plasma cells.

The spleen. The spleen in *Chimaera monstrosa* comprises about 1% of the body weight (Fänge, 1977). A mosaic of red and white areas observed on the surface might be interpreted as an occurrence of red and white pulp. In histological sections, lymphoid areas are found immediately under the connective tissue capsule and in small numbers within the parenchyma. These cell masses have diffuse outlines and can hardly be compared with the white pulp (Malpighian bodies) found in the mammalian spleen. Previous light microscopic studies of the structure and the cell contents of the *Chimaera* spleen made by Scatizzi (1932) and by Fänge and Sundell (1969) stress the great number of erythrocytes in different developmental stages, which suggests that the spleen is an important erythrocytopoietic organ. The same conclusion also can be drawn from the large number of erythrocytes and erythroblasts in mitosis







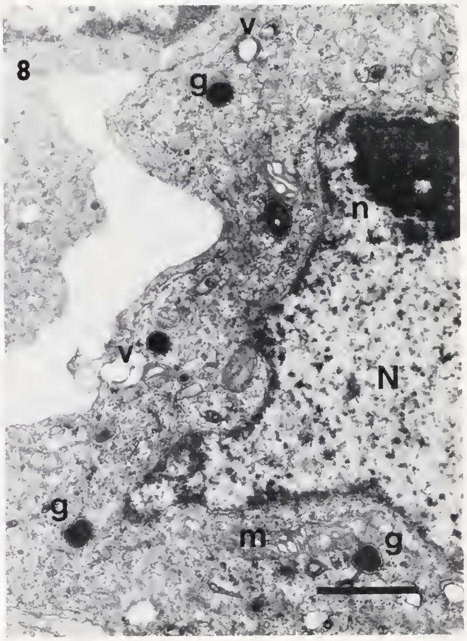


FIGURE 5. Heterophil from the orbital organ. Horseshoe-shaped nucleus (N) with marginal heterochromatin. A great diversity in the appearance of the cytoplasmic granules, several of them with electron-lucent central areas. Solitary dense rods (r), mitochondria (m) and pseudopodia (p). Fixation as in Figure 1. Bar: 1 μm.

FIGURE 6. Low magnification of a portion of the orbital organ, showing heterophils (H) and one single lymphocyte (L). The diversity in structure of the granules and in the form of the nuclei is obvious.

found in the areas studied by electron microscopy (Fig. 11). The leucocytes constitute a minority. Among the leucocytes those of monocyte type predominate but there are also blast cells, granulocytes, and macrophages. The monocytes have their sparse, characteristic granules about 0.3 μm in length and with a thin halo. Macrophages mostly have cytoplasmic remnants of engulfed cells. No plasma cells were observed.

DISCUSSION

In Elasmobranchii, as well as in the holocephalan fish, *Chimaera monstrosa,* most lymphomyeloid tissues are located in regions unlike those of higher vertebrates (Fänge, 1984). In elasmobranchs the major lymphomyeloid tissues are found in the oesophagus and/or constitute an epigonal organ. In *Chimaera* similar tissues are located within cavities of the cranial skeleton. The majority of the cells of the lymphomyeloid tissues of *Chimaera* are structurally and ultrastructurally similar to leukocytes of higher vertebrates and a corresponding classification and terminology of the cells can be used. The high diversity in cell equipment and the occurrence of several morphological points of concordance with higher vertebrates indicate that *Chimaera* has developed a variety of immunological functions. For elasmobranchs this has been demonstrated experimentally by Good *et al.* (1966), Hildemann (1970), Sigel (1974), and Litman *et al.* (1976), among others.

The thymus of *Chimaera* is well developed and is equipped with cells similar to those found in the thymus of higher vertebrates. In addition to the predominating small lymphocytes, the thymus contains large cells of type macrophages, interdigitating cells, and reticulocytes. The latter are in particularly close contact with, and infiltrate into, the peripheral portions of the lymphocytes and may influence the differentiation of the thymocytes. Such a regulatory role in the production of thymic lymphocytes has been reported from studies on several vertebrates (*e.g.*, Rappay *et al.*, 1971; Rouse *et al.*, 1979; Duijvestijn and Hoefsmit, 1981). Although the cells of the thymus of *Chimaera* are ultrastructurally compatible with those of other vertebrates, nothing can be said about a functional diversity comparable to that found in vertebrates.

As reported by Fey (1966), the granules of granulocytes from lower vertebrates show a high diversity as to form and electron density. In *Chimaera,* however, there are no difficulties in distinguishing two main groups of granulocytes, eosinophils and heterophils, which can be identified histologically as well as in the electron microscope.

The large spherical granules in the cytoplasm of eosinophils have been identified as lysosomes (Cohn and Wiener, 1963). This has been further supported by Fänge (1968) who showed that the granules concentrate neutral red and suggested that they are supplied with hydrolytic enzymes. The homogeneously electron-dense granules lack a central crystalloid core. This corresponds to results from several lower vertebrates (Kelényi and Németh, 1969; Homma *et al.*, 1984), however, mammals usually

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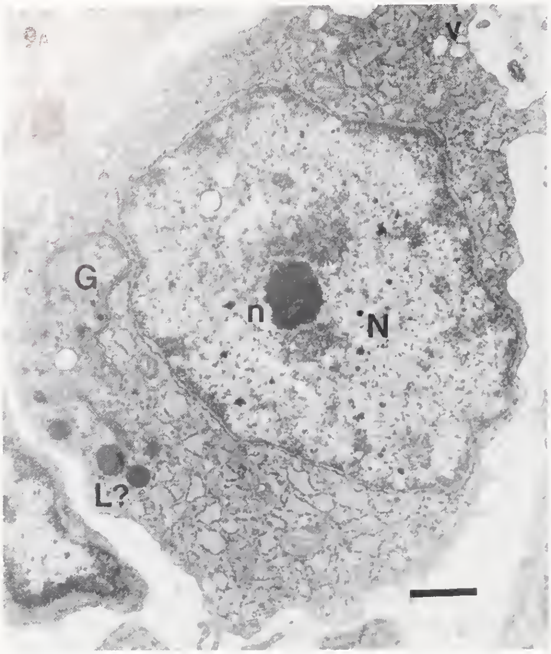
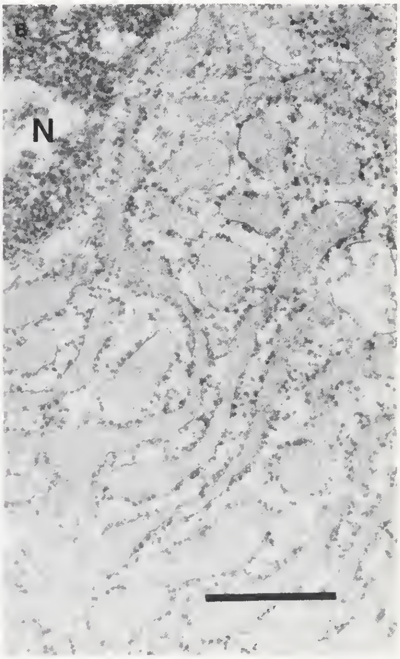
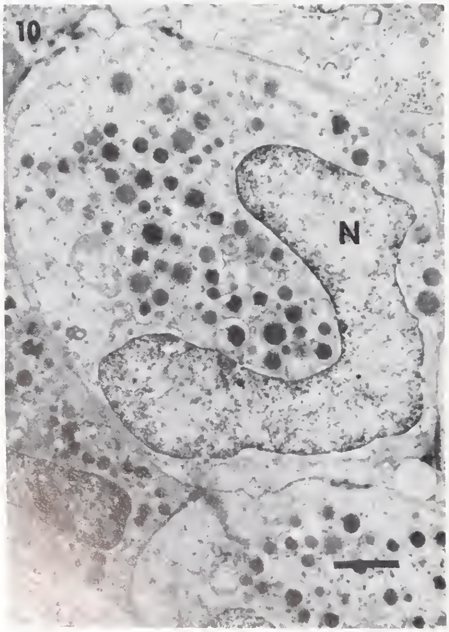
Generally a marginal orientation of the heterochromatin. Some heterophils with pseudopodia (p). Fixation as in Figure 1. Bar: 3 μm.

FIGURE 7. The orbital organ. Portion of a heterophil. The granules are round or have an irregular outline. Two grades of density occur within most granules. The "empty" region occurs within the dense central region while the outer, less dense area appears granular (arrows). Fixation as in Figure 2. Bar: 1 μm.

FIGURE 8. Portion of a monocyte from the orbital organ. Solitary characteristic granules (g) supplied with thin halos. Some vacuoles (v) and mitochondria (m). Nucleus (N) with a marked nucleolus (n). Fixation as in Figure 2. Bar: 1 μm.







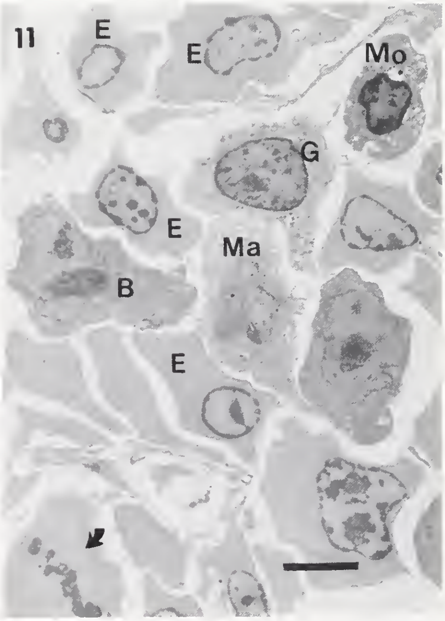


FIGURE 9a. Plasma cell from the orbital organ. Nucleus (N) with a marked nucleolus (n) and scattered heterochromatin. Cytoplasm with the characteristic well-developed rough endoplasmic reticulum, which here forms cisternae. The Golgi complex (G) is prominent. Some lysosome-like bodies (L?) and vacuoles (v) in the cytoplasm.

have a central crystalline structure in their eosinophilic granules (Bargmann and Knoop, 1958; Braunsteiner and Pakesch, 1957).

The heterophils of *Chimaera* show an obvious structural variation, especially in the granules. In the same cell as well as when comparing cells, granules show a marked difference in size, form, and content. Several granules have central regions which show a deviating density, but crystalline inclusions are rare. "Empty" areas often occupy the internal parts of the granules. Such regions, appearing like holes or excavations, may also protrude into the cytoplasm. Similar structures have been reported in amphibians and reptiles and interpreted as signs of an increased interaction between the granules and the cytoplasm (Kelényi and Németh, 1969). Since such "empty" vesicles occur peripherally in the cells, probably in connection with endocytosis, the granules might be interpreted as lysosomes engulfing "empty" endocytotic vesicles. However, there may be another explanation: some of the compact granules have especially electron-dense central regions (arrow in Fig. 7), and a peripheral frame of this dense material often occurs around the central "empty" region (arrow in Fig. 7). This may indicate that the dense material is easily damaged and dissolved during the preparation of the tissue, or that a disappearance of dense material is caused by the electron beam.

The granules of the granulocytes seem to originate at the Golgi region. This is especially obvious for the eosinophils which show a gradual increase in size and density of the granules from the Golgi region towards the periphery of the cells. The great amount of rough endoplasmic reticulum in the peripheral parts of the cells may be correlated to the increased density of the granules and to their suggested increased contents of proteolytic enzymes. The even outlines of the eosinophils give no indication of an endo- or exocytosis and thus no intimation of where or when the enzymes are active.

In the lymphomyeloid tissues of the cranial skeleton of *Chimaera,* granulocytes predominate but often occur near groups of small lymphocytes. The presence of some intermediate forms between lymphocytes and granulocytes points to lymphocyte-like cells as possible stem cells of the granulocytes. The occurrence of blast cells and the high concentration and structural variation of granulocytes in the orbital and suprapalatal tissues indicate that these tissues are main localities for granulocytopoiesis. A granulocytopoietic function seems to be predominant in the cranial lymphomyeloid tissues of *Chimaera* and, like the corresponding lymphomyeloid organs of elasmobranchs, they might be designated "white marrow" or "cartilage marrow" to distinguish them from the mainly haemopoietic red bone marrow of higher vertebrates (Kolmer, 1923).

Since the work by Fagraeus (1948), it has been possible to correlate, in mammals, the formations of antibodies with the occurrence of plasma cells. In the rabbit fish, *Chimaera,* the orbital and suprapalatal tissues hold cells, scattered among the pre-

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FIGURE 9b. Higher magnification of a portion of a plasma cell showing numerous cisternae formed by the rough endoplasmic reticulum and their moderately electron-dense interior. Nucleus (N). Fixation as in Figure 1. Bar in 9a: 1 μm. Bar in 9b: 0.5 μm.

FIGURE 10. Heterophil from the suprapalatal region. The cytoplasmic granules are comparatively small and spherical and lack central "empty" zones. The whole cell appears faintly electron-dense. Horseshoe-shaped nucleus (N). Fixation as in Figure 2. Bar: 1 μm.

FIGURE 11. A survey of cells from the spleen. Erythrocytes (E) predominate. Other cell types: macrophage (Ma), monocyte-like cell (Mo), granulocyte (G) and blast cell (B). At arrow a mitosis. Fixation as in Figure 1. Bar: 5 μm.

dominating granulocytes and which have the ultrastructure of plasma cells. Morphologically they deviate in two respects from the plasma cells of higher vertebrates: their heterochromatin does not show an arrangement of radial strings and their rough endoplasmic reticulum forms a number of cistern-like structures. Otherwise, the rough endoplasmic reticulum shows an abundance and an arrangement that strongly suggest these cells to be plasma cells with their comprehensive protein synthesis. As shown by Fänge and Sundell (1969), the blood plasma of *Chimaera* contains two or three protein fractions migrating towards the cathode like the gammaglobulins of higher vertebrates. In another holocephalan fish, *Callorhynchus callorhynchus,* Sánchez *et al.* (1980) showed the presence of an IgM-like hemagglutinin. This macromolecule has been further studied by Garrido and De Ioannes (1981) by using electron microscopy. They suggested a pentameric structure similar to that reported for human IgM. However, the identification of antibodies in *Chimaera monstrosa* as well as of cells performing an antibody synthesis must await further research.

ACKNOWLEDGMENTS

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THE ACCUMULATION AND DISTRIBUTION OF VANADIUM, IRON, AND MANGANESE IN SOME SOLITARY ASCIDIANS

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ABSTRACT

The vanadium, iron, and manganese contents of 15 species of solitary ascidians belonging to the suborders Phlebobranchia and Stolidobranchia were determined by thermal neutron activation analysis. Vanadium was detectable in all species examined. In general, the vanadium content in various tissues of the Phlebobranchia was considerably higher than the iron and manganese contents. The blood cells especially contained a large amount of vanadium. The highest value (21 μg vanadium/mg dry weight) was obtained from blood corpuscles of *Ascidia ahodori*. Species in the suborder Stolidobranchia, on the other hand, had smaller quantities of vanadium in comparison with those in the suborder Phlebobranchia. The iron and manganese contents did not differ greatly between the two suborders. The data are considered in the light of physiological roles of these transition metals in ascidians.

INTRODUCTION

The ability of many species of ascidians to concentrate vanadium and other metals from seawater is one of the physiological peculiarities which distinguishes these organisms from other classes of animals. Recent data give concentrations of dissolved vanadium in seawater as about 35 n*M* (Cole *et al.*, 1983; Collier, 1984), while the concentration of vanadium in ascidian blood cells can be as high as 0.15 *M*, a value about seven orders of magnitude higher (Macara *et al.*, 1979a).

In studies with *Phallusia mammillata,* Henze (1911) was the first to find that ascidian blood contained unexpectedly high amounts of vanadium. Many investigators subsequently analyzed metal content in species belonging to all three suborders (the Aplousobranchia, Phlebobranchia, and Stolidobranchia). The resulting data have shown generally that several species in the suborder Aplousobranchia have a high vanadium content, and that significant vanadium is likewise found in representatives of the Phlebobranchia, whereas species in the suborder Stolidobranchia contain relatively smaller amounts of vanadium, but a high iron content (Goodbody, 1974; Swinehart *et al.,* 1974; Biggs and Swinehart, 1976; Hawkins *et al.,* 1983). Little more information has previously been available on the vanadium accumulated in the species of the Stolidobranchia, however, because of the detection limits of analytical methods used in former experiments (Swinehart *et al.,* 1974; Danskin, 1978; Macara *et al.,* 1979a). The present studies were designed to fill this void in our knowledge of metal accumulation by stolidobranchial species and simultaneously to identify particular vanadium-rich tissues of the species of Phlebobranchia using neutron acti-

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vation analysis. The data are transformed to molar concentrations per unit of tissue for consideration of the roles of these metals in ascidian physiology.

MATERIALS AND METHODS

Species were collected at the following locations. *Ciona savignyi**, *Ascidia ahodori, A. sydneiensis samea, Chelyosoma siboja, Styela plicata,* and *Halocynthia roretzi:* the Asamushi Marine Biological Station, Tohoku University on the Bay of Mutsu, Japan; *Polycarpa cryptocarpa* and *Pyula sacciformis:* the Noto Marine Laboratory, University of Kanazawa on the Inlet of Tsukumo, Japan; *H. aurantium:* Hakodate Aquarium on the Bay of Hakodate, Japan; *Molgula manhattensis:* the Nakajima Marine Biological Station, Ehime University on the Strait of Sekido, Japan; *C. intestinalis**, *A. malaca, Phallusia mammillata, H. papillosa,* and *Microcosmus sulcatus:* the Stazione Zoologica di Napoli on the Gulf of Naples, Italy.

At least five specimens of each species were analyzed for metals with the exception of *M. manhattensis,* of which three specimens were examined. Each specimen was cleaned of extraneous materials. Blood was obtained via heart puncture, whereupon plasma and corpuscles were separated by centrifuging the blood for 10 min at 2000 × *g*. The test, mantle, branchial basket, stomach, liver, and gonads were rinsed three times with filtered seawater. All tissues were weighed wet before they were dried in porcelain crucibles in a drying oven at 110°C to a constant dry weight per sample. Samples (about 20 mg dry weight) sufficient for analysis of metals by means of neutron activation analysis, were mineralized in a muffle furnace at 500°C for 24 h. The ash was dissolved in 5.0 ml of 0.1 N HNO_3 (super special grade, Wako Pure Chemical Indust. Ltd., Japan) and put into a polyethylene capsule. For applying the neutron activation analysis to measuring the amount of vanadium and manganese, each sample was irradiated for 2 min in the TRIGA MARK II nuclear reactor at Rikkyo University with thermal neutrons having a flux of 5×10^{11} $n/cm^2 \cdot s^{-1}$. The radioactivity of ^{52}V produced in the irradiated sample was measured with a 50-cm^3 Ge(Li) γ-ray spectrometer (Canberra Inc.) 2 min after irradiation. Sixty min after irradiation, the radioactivity of ^{56}Mn was measured in the same manner as described above to avoid the interference of ^{27}Mg produced in the irradiated sample. The photon energies of ^{52}V and ^{56}Mn used for measurement were 1432 KeV and 846 KeV, respectively. The amounts of vanadium and manganese were determined by comparing the γ-ray spectrograms with those of standard samples. Smoothing of spectral data and calculation of the peak area were done by the method proposed by Adams and Dams (1970) employing an automatic computer system. This analytical method has already been verified to be a most sensitive method of detection of vanadium in the ascidian (Papadopoulou and Kanias, 1977; Michibata, 1984).

After the radionuclides were allowed to decay to negligible concentrations, aliquots of each sample were submitted for analysis of iron by atomic absorption spectrometry (Equipment: Hitachi GA-2 flameless atomic absorption spectrometer). The absorption line used was 3719.9 nm.

RESULTS

The vanadium, iron, and manganese concentrations for different tissues of the chosen species appear in Tables I and II. Table I shows data for seven species belong-

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* The species, *C. robusta,* used in our previous papers (Hori and Michibata, 1981; Michibata, 1984; Michibata *et al.,* 1985) recently was renamed *C. intestinalis* by Hoshino and Nishikawa (1985). It inhabits European waters. With this renaming, the other species, *C. intestinalis sensu* Hoshino and Tokioka 1967 was also renamed *C. savignyi.* This taxonomic problem is examined in detail in their paper.

TABLE I

Metal contents in tissues of Phlebobranchiata (ng/mg dry weight)

Species	Vanadium	Iron	Manganese
Ciona intestinalis			
Tunic	1.7 ± 0.4	370.0 ± 39.4	188.8 ± 78.1
Mantle	338.4 ± 23.8	148.2 ± 11.4	29.3 ± 5.4
Branchial basket	337.5 ± 47.8	251.4 ± 27.6	69.9 ± 7.3
Stomach	163.2 ± 14.6	294.7 ± 69.0	36.4 ± 8.1
Liver	—	—	—
Gonad	100.9 ± 7.5	65.8 ± 8.9	77.0 ± 14.9
Corpuscles	330.7 ± 14.1	424.4 ± 49.4	31.4 ± 9.0
Plasma	0.4 ± 0.2	89.5 ± 4.8	9.2 ± 1.9
Ciona savignyi			
Tunic	1.2 ± 0.5	87.6 ± 6.5	4.8 ± 1.5
Mantle	333.0 ± 39.7	54.8 ± 7.3	7.4 ± 0.9
Branchial basket	1037.5 ± 109.2	173.8 ± 24.3	17.3 ± 0.8
Stomach	507.4 ± 69.6	58.8 ± 8.0	20.2 ± 3.1
Liver	—	—	—
Gonad	143.6 ± 23.9	54.7 ± 5.3	13.2 ± 1.1
Corpuscles	1577.1 ± 338.2	78.3 ± 5.1	107.6 ± 13.8
Plasma	12.9 ± 0.4	119.6 ± 4.8	15.4 ± 1.9
Ascidia malaca			
Tunic	85.8 ± 3.8	1683.7 ± 194.3	392.1 ± 14.2
Mantle	645.6 ± 211.4	494.3 ± 22.1	17.9 ± 2.1
Branchial basket	737.3 ± 193.8	1204.9 ± 127.5	21.8 ± 4.0
Stomach	—	—	—
Liver	282.1 ± 43.2	275.6 ± 49.1	25.5 ± 7.3
Gonad	—	—	—
Corpuscles	4446.2 ± 509.6	741.5 ± 96.2	37.0 ± 3.4
Plasma	—	41.8 ± 5.0	—
Ascidia ahodori			
Tunic	1229.7 ± 142.8	356.7 ± 44.0	9.1 ± 2.5
Mantle	5723.1 ± 614.4	563.7 ± 73.9	2.3 ± 0.8
Branchial basket	6555.0 ± 331.2	480.6 ± 44.5	3.2 ± 0.6
Stomach	743.9 ± 159.1	135.9 ± 21.3	1.0 ± 0.8
Liver	1204.8 ± 122.8	241.1 ± 29.7	0.2 ± 0.2
Gonad	—	—	—
Corpuscles	21120.9 ± 1985.7	1702.1 ± 222.2	34.3 ± 1.6
Plasma	522.2 ±	—	—
Ascidia sydneiensis samea			
Tunic	30.6 ± 2.6	1432.6 ± 291.1	188.4 ± 37.2
Mantle	333.9 ± 46.1	276.6 ± 19.1	7.0 ± 0.9
Branchial basket	730.4 ± 95.2	299.0 ± 46.4	12.8 ± 0.8
Stomach	—	—	—
Liver	163.3 ± 19.3	140.3 ± 26.4	7.2 ± 0.5
Gonad	339.9 ± 22.1	310.5 ± 56.4	15.2 ± 4.6
Corpuscles	4675.9 ± 353.5	904.7 ± 185.6	26.8 ± 5.5
Plasma	27.6 ± 4.5	114.3 ± 10.8	20.1 ± 0.7
Phallusia mammillata			
Tunic	15.2 ± 1.8	117.7 ± 15.6	4.8 ± 0.8
Mantle	442.9 ± 106.9	127.0 ± 29.4	16.6 ± 3.4
Branchial basket	1502.3 ± 86.1	147.0 ± 50.6	29.3 ± 3.2
Stomach	—	—	—
Liver	136.6 ± 17.5	51.1 ± 11.0	24.5 ± 2.7
Gonad	—	—	—
Corpuscles	9859.9 ± 1780.7	413.4 ± 111.9	18.9 ± 1.0
Plasma	—	92.7 ± 17.8	7.2

TABLE I (*Continued*)

Species	Vanadium	Iron	Manganese
Chelyosoma siboja			
Tunic	6.0 ± 1.3	283.8 ± 37.5	24.7 ± 4.7
Mantle	39.4 ± 2.8	348.5 ± 50.0	30.1 ± 3.4
Branchial basket	1732.4 ± 138.9	471.7 ± 14.5	44.2 ± 5.5
Stomach	502.8 ± 34.0	511.8 ± 48.0	35.4 ± 6.7
Liver	330.5 ± 25.4	634.9 ± 35.2	38.5 ± 6.0
Gonad	—	—	—
Corpuscles	8.2 ± 0.8	138.1 ± 37.4	18.2 ± 1.2
Plasma	—	58.8 ± 5.3	—

Data are expressed as means ± standard errors. It was difficult to determine the metal contents in blood plasma because of the severe interference of γ-rays of ^{24}Na produced by the irradiation with thermal neutrons. Therefore, these data were not available in some cases.

ing to the suborder Phlebobranchia. All the species had a large vanadium content in their blood cells and branchial basket, with the exception of *Chelyosoma siboja*, in which the blood cells had a relatively small quantity of vanadium while the branchial basket had a large amount. The highest value for vanadium (21120.9 ng/mg dry weight) was obtained from the blood cells of *Ascidia ahodori*. Representative levels of iron and manganese, on the other hand, were 1702.1 ng/mg dry weight in blood cells of *A. ahodori* and 392.1 ng/mg dry weight in the tunic of *A. malaca*, respectively. In general, the vanadium content in various tissues of the Phlebobranchia was considerably higher than the iron and manganese contents. The blood cells in particular contained a large excess of vanadium over iron and manganese.

Table II lists the metal content in eight species belonging to the suborder Stolidobranchia. Vanadium was detectable in almost all tissues of these species; however, the quantity was small in comparison to that in the Phlebobranchia. For example, a vanadium content of 17.9 ng/mg dry weight, obtained in the tunic of *Molgula manhattensis*, was the highest, a level less than one-thousandth by weight of that in the blood cells of *A. ahodori* (Table I). Iron and manganese levels, on the other hand, did not differ greatly between the two suborders. The data revealed a tendency for the iron to be accumulated in the blood cells and manganese in the tunic.

Molar concentrations of vanadium, iron, and manganese in living tissues were then determined for tissues having 90% or greater moisture content. Figure 1 expresses the molar concentrations for all three metals from four species: *A. ahodori*, *A. sydneiensis samea*, *Styela plicata*, and *Halocynthia roretzi*, with two species each for each of the two suborders (Tables I, II). Concentrations of more than 10 μM vanadium were found even in the tunic of both *A. ahodori* and *A. sydneiensis samea*, while considerably lower vanadium concentrations (ranging from 0.1 to 10 μM) were measured in almost all tissues of the Stolidobranchia. *A. ahodori* had in excess of 10 mM vanadium in the mantle, branchial basket, and blood corpuscles. By contrast, the vanadium levels were only 0.1 to 1 μM in the tunic, mantle, branchial basket, and blood cells of *S. plicata*. Like others before us, therefore, we find large differences in vanadium concentration among ascidian species. In this study, however, we document for the first time the certain presence of vanadium in species of the Stolidobranchia, as well as the presence of only relatively smaller differences between species in iron and manganese contents, which range from 10 to 100 μM and 1 to 10 μM, respectively, for all species examined.

TABLE II

Metal contents in tissues of Stolidobranchiata (ng/mg dry weight)

Species	Vanadium	Iron	Manganese
Polycarpa cryptocarpa var. kuroboja			
Tunic	6.0 ± 0.8	1709.6 ± 114.8	96.0 ± 12.9
Mantle	1.2 ± 0.01	79.6 ± 5.6	6.2 ± 0.7
Branchial basket	1.5 ± 0.3	117.0 ± 11.1	6.8 ± 0.4
Stomach	3.2 ± 0.7	44.7 ± 1.5	9.8 ± 2.1
Liver	—	—	—
Gonad	—	—	—
Corpuscles	13.4 ± 2.9	2755.5 ± 152.6	63.0 ± 5.0
Plasma	1.0 ±	151.0 ± 10.9	16.7 ± 1.0
Styela plicata			
Tunic	2.6 ± 0.5	289.4 ± 20.3	38.1 ± 4.4
Mantle	0.3 ± 0.03	43.3 ± 2.1	3.5 ± 1.1
Branchial basket	0.5 ± 0.1	139.1 ± 23.2	11.4 ± 1.2
Stomach	0.4 ± 0.2	72.1 ± 4.0	6.8 ± 1.5
Liver	—	—	—
Gonad	1.3 ± 0.3	67.4 ± 6.3	6.6 ± 0.7
Corpuscles	3.5 ± 0.5	1095.0 ± 117.6	31.9 ± 5.8
Plasma	1.3 ± 0.4	176.3 ± 10.2	3.7 ± 1.1
Pyura sacciformis			
Tunic	1.0 ± 0.2	318.5 ± 13.2	230.4 ± 37.1
Mantle	0.6 ± 0.2	87.5 ± 12.8	9.2 ± 0.9
Branchial basket	1.1 ± 0.2	147.1 ± 6.0	20.4 ± 3.0
Stomach	1.1 ± 0.03	140.4 ± 1.0	26.0 ± 2.4
Liver	—	—	—
Gonad	1.0 ± 0.5	256.4 ± 4.4	18.8 ± 3.3
Corpuscles	0.6 ± 0.2	451.4 ± 61.8	25.0 ± 2.2
Plasma	2.0 ± 1.0	93.8 ± 6.9	3.4 ± 0.7
Halocynthia roetzi			
Tunic	5.8 ± 2.5	50.6 ± 13.3	28.4 ± 2.7
Mantle	0.5 ± 0.07	14.7 ± 1.4	3.1 ± 0.2
Branchial basket	1.9 ± 0.5	45.9 ± 8.4	11.0 ± 0.7
Stomach	—	—	—
Liver	2.5 ± 1.0	136.3 ± 22.2	11.5 ± 0.9
Gonad	2.1 ± 1.3	50.7 ± 8.0	7.5 ± 0.8
Corpuscles	3.6 ± 0.4	320.0 ± 25.0	40.2 ± 6.8
Plasma	0.6 ±	104.8 ± 5.3	19.8 ± 3.5
Halocynthia aurantium			
Tunic	1.3 ± 0.2	83.1 ± 8.1	31.1 ± 3.3
Mantle	0.9 ± 0.1	76.1 ± 19.4	3.7 ± 0.4
Branchial basket	0.8 ± 0.1	155.2 ± 18.4	14.8 ± 0.2
Stomach	2.5 ± 0.5	176.0 ± 19.4	30.1 ± 3.1
Liver	3.5 ± 0.2	352.1 ± 31.0	19.5 ± 0.7
Gonad	0.3 ± 0.04	76.3 ± 16.2	14.3 ± 1.1
Corpuscles	2.2 ± 0.2	245.3 ± 18.9	38.5 ± 3.4
Plasma	0.02 ± 0.003	127.8 ± 19.0	1.4 ± 0.7
Halocynthia papillosa			
Tunic	1.0 ± 0.4	43.5 ± 3.9	19.1 ± 1.0
Mantle	2.3 ± 0.5	61.4 ± 3.0	20.1 ± 0.7
Branchial basket	2.9 ± 1.0	62.0 ± 2.5	39.7 ± 9.2
Stomach	—	—	—
Liver	2.9 ± 0.3	114.9 ± 8.7	112.2 ± 4.6
Gonad	0.9 ± 0.2	45.5 ± 0.6	38.6 ± 0.3
Corpuscles	7.7 ± 1.6	640.0 ± 41.2	188.9 ± 8.7
Plasma	0.9 ± 0.2	54.0 ± 3.1	65.9 ± 4.1

TABLE II (*Continued*)

Species	Vanadium	Iron	Manganese
Microcosmus sulcatus			
Tunic	0.3 ± 0.01	72.4 ± 12.4	7.9 ± 0.5
Mantle	0.1 ± 0.03	68.4 ± 1.3	11.5 ± 1.3
Branchial basket	0.6 ± 0.4	84.4 ± 4.3	90.8 ± 10.4
Stomach	—	—	—
Liver	1.7 ± 0.3	49.5 ± 2.8	54.9 ± 5.9
Gonad	0.1 ± 0.01	49.9 ± 6.8	30.6 ± 1.8
Corpuscles	13.9 ± 8.0	791.2 ± 83.9	226.9 ± 4.1
Plasma	—	52.8 ± 1.7	27.1 ± 1.7
Molgula manhattensis			
Tunic	17.9 ±	7588.3 ±	534.6 ±
Mantle	1.5 ±	155.3 ±	26.9 ±
Branchial basket	1.5 ±	1041.7 ±	10.1 ±
Stomach	0.9 ±	240.4 ±	27.6 ±
Liver	—	—	—
Gonad	1.2 ±	8.7 ±	7.5 ±
Corpuscles	2.6 ±	125.0 ±	37.4 ±
Plasma	0.2 ±	105.7 ±	10.0 ±

See footnote in Table I. As to *M. manhattensis*, three specimens were submitted.

DISCUSSION

The data shown in Tables I and II agree substantially with previous reports that species belonging to the suborder Phlebobranchia have a large vanadium content, while those in the suborder Stolidobranchia have a smaller amount (Swinehart *et al.*, 1974; Biggs and Swinehart, 1976; Danskin, 1978; Macara *et al.*, 1979a). High levels of vanadium have previously been found in the family Ascidiidae; *A. nigra* was known to contain a high concentration of vanadium in its blood cells at 26.8 μg/mg dry weight (Macara *et al.*, 1979a). The present studies likewise show that other species in this family accumulate especially high levels of vanadium; *A. ahodori* had a comparable amount of vanadium in its blood cells at 21.1 μg/mg dry weight (Table I). In *Phallusia mammillata*, the original species in which ascidian vanadium was found (Henze, 1911), we reconfirm the presence of a high amount of vanadium in the blood cells. In addition, species belonging to the suborder Phlebobranchia were found in our experiments to concentrate a relatively high amount of vanadium in other tissues as well. Whether these are measures of actual tissue content of vanadium, or result from the presence in the different tissues of contaminating blood cells (the tissues were not perfused after dissection) remains to be determined. Because cell types other than blood cells (*e.g.*, egg test cells; Hori and Michibata, 1981) contain vanadium, a careful analysis of specific cell types for metal content now seems justified.

It is important to note that significant levels of vanadium were detected in all tissues of species belonging to the suborder Stolidobranchia (Table II). In contrast with former studies, our experiments using thermal neutron activation analysis are the first to reveal that species in this suborder without doubt contain vanadium. In previous reports based on colorimetric analysis (Ciereszko *et al.*, 1963; Danskin, 1978), atomic absorption spectrometry (Botte *et al.*, 1979; Hawkins *et al.*, 1983) and plasma emission spectrometry (Macara *et al.*, 1979a; Hawkins *et al.*, 1980), the detection limits for vanadium were greater than 20 ppm; therefore, the vanadium content in the species of the suborder Stolidobranchia could not be determined with certainty.

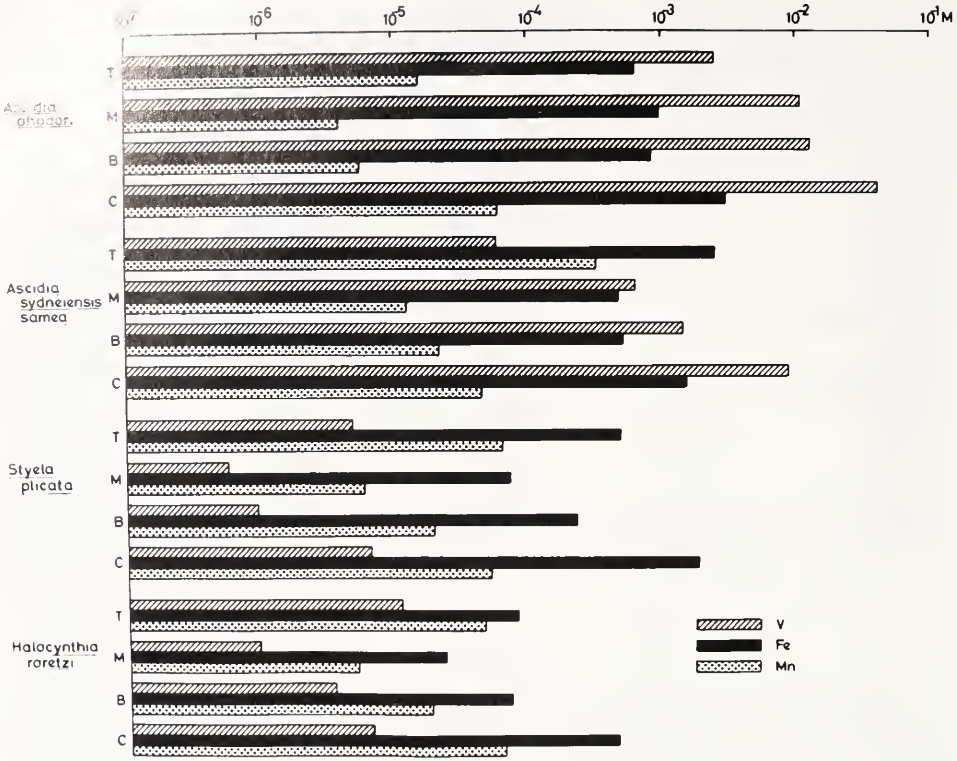

FIGURE 1. Molar concentrations of vanadium, iron, and manganese contained in each ascidian tissue. *A. ahodori* and *A. sydneiensis samea* are representatives of Phlebobranchiata and *S. plicata* and *H. roretzi* are of Stolidobranchiata. T = Tunic, M = Mantle, B = Branchial basket, C = Corpuscles.

As shown in Table II, neutron activation analysis allowed detection of the low, but significant, levels of vanadium (several hundred ppb in certain tissues) for these species. These concentrations are about one hundred times those of comparable mammalian tissues (Söremark, 1967). It appears, therefore, that mechanisms for vanadium accumulation are intact in the Stolidobranchia, leaving open the possibility that ascidians of the suborder Stolidobranchia represent transitional forms with regard to physiological mechanisms involving vanadium.

The data appearing in Tables I and II also show that the contents of iron and manganeses in general do not vary sharply between the two suborders studied, in contrast with vanadium content, although some differences between tissues are seen. The largest amounts of iron (7588.3 ng/mg dry weight) and manganese (534.6 ng/mg dry weight) were found in the tunic of *M. manhattensis;* however, the tunic did not contain high levels of either metal in other species. The somewhat higher iron content (16.4 to 26.6 μg/mg organic dry weight) in freshly collected *M. manhattensis* previously reported by Swinehart *et al.*, (1974) most likely resulted from ingested particulate matter from the collection area. Data from other studies of manganese content in ascidians (Noddack and Noddack, 1940; Carlisle, 1968; Swinehart *et al.*, 1974; Botte *et al.*, 1979) agree generally with our analytical findings (Tables I, II).

Webb (1939) first pointed out that high concentrations of vanadium in ascidian species correlated with certain evolutionary traits of the class Ascidiacea; he also predicted that it would be a primitive characteristic which had been lost in species of the more specialized Stolidobranchia. Subsequent analysis by many investigators confirmed that, in general, species from the suborders Aplousobranchia and Phlebobranchia had a large vanadium content, whereas species from the suborder Stolidobranchia contained a smaller quantity of vanadium, while retaining large quantities of iron (Goldberg *et al.,* 1951; Levine, 1961; Ciereszko *et al.,* 1963; Carlisle, 1968; Macara *et al.,* 1979a). Furthermore, based on evidence that the iron content of the Aplousobranchs *Eudistoma mole* and *Distaplia occidentalis* was of the same order of magnitude as the vanadium content, Swinehart *et al.* (1974) suggested that these species might indeed represent animals that were in transition between the vanadium and iron "users." However, until more species are submitted for detailed analysis of their metal content by methods as sensitive as the ones used in the present study, we cannot conclude that the relative concentrations of the two metals in different ascidian subfamilies reflects phylogeny.

Figure I shows that, although relatively narrow diversities in tissue iron and manganese concentrations were observed between different tissues, there is a very wide diversity in the vanadium concentrations, ranging from 0.1 μM to 10 mM. This finding may be quite meaningful in considerations of the physiological roles played by vanadium in ascidians. The present data indicate that the largest amounts of vanadium are accumulated in blood cells and/or the branchial basket, which contains large foci of blood cells. It is possible, therefore, that the dominant cell types accumulating this metal are blood cells, a finding that agrees substantially with studies by others (Macara *et al.,* 1979a). Because a respiratory function of blood cell vanadium (as an oxygen carrier, Carlisle, 1968) has been eliminated (Macara *et al.,* 1979b), we must consider other possibilities for adaptive functions of transition metal accumulation in blood cells.

Recent X-ray microanalysis studies with blood cells of vanadium-accumulating ascidians have demonstrated that the metal is concentrated in cytoplasmic vacuoles (Botte *et al.,* 1979; Scippa *et al.,* 1982). Our own previous studies have revealed similarly that considerable amounts of vanadium and iron are detectable in condensed masses of granules in the oocyte test cells of unfertilized eggs of *Ciona intestinalis* (Hori and Michibata, 1981). For selective localization in the vacuoles of specialized cells, it would seen likely that both metals are accumulated through the plasma membrane by a specialized transport system. Kustin and his co-workers have recently provided evidence for a specific vanadate transport system in the plasma membranes of ascidian blood cells (Dingley *et al.,* 1981). They likewise have described a yellow blood pigment, which they named tunichrome, which occurs within the same vacuoles as the vanadium, and which appears to reduce vanadium(V) to vanadium(IV) (Gilbert *et al.,* 1977; Macara *et al.,* 1979a, b; Macara; 1980; Agudelo *et al.,* 1982; Robinson *et al.,* 1984). The chemical formula and structure of tunichrome (Tunichrome B-1) from the blood cells of *A. nigra* has been described by Bruening *et al.* (1985). Robinson *et al.* (1986) have shown that tunichrome may be involved in tunic formation in ascidian embryos. On the other hand, Rowley (1983) has suggested that vanadium and its associated compounds may be part of the antimicrobial armoury of ascidian blood cells. The clear presence of vanadium in the blood cells of all ascidian species, documented for the first time in this study, strongly suggests that, whatever the physiological role played by vanadium in ascidians, it has not been lost in any of the extant species. Studies of the relative contributions of blood cell vanadium, iron, and manganese to *in vitro* antimicrobial chemical reaction systems and/or tunic formation systems may allow description of the relationship between these transition metals in adaptive functions carried out by blood cells.

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UPTAKE OF AMINO ACIDS BY *PAREURYTHOE CALIFORNICA*: SUBSTRATE INTERACTION MODIFIES NET INFLUX FROM THE ENVIRONMENT

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ABSTRACT

Pareurythoe californica is capable of simultaneous net uptake of 18 amino acids, each present at an initial concentration of 200 nM. Rates of uptake are comparable for all amino acids tested. Kinetics of uptake are well described by the Michaelis-Menten equation. Neither bacteria nor other epifauna play a significant role in the observed uptake.

The net entry of the amino acids tested was inhibited in the presence of equimolar concentrations of representatives of all major classes of amino acids (*i.e.*, polar and nonpolar neutral, dicarboxylic, polybasic) at concentrations comparable to those found in the environment. Thus, a mixture mimicking the composition and concentration of substrates normally present in the environment was used to obtain a more realistic estimate of entry rates under natural conditions. When this was done, the contribution of exogenous amino acids to the nitrogen needs (based on ammonia excretion) and the requirement for reduced carbon (based on oxygen consumption) of *Pareurythoe* ranged from 10% to 50%, depending on levels of substrate available in the environment.

INTRODUCTION

High performance liquid chromatography (HPLC) has been used to demonstrate net uptake of amino acids by bacteria-free marine organisms (Manahan *et al.*, 1983; Davis and Stephens, 1984a, b; Lu Ming and Stephens, 1984). However, such work has often involved mixtures of amino acids which bear little resemblance to those found in the natural habitat of the organisms concerned. Since amino acids may interact during transport, the use of arbitrary mixtures as a basis for estimation of the total net entry from the environment is open to question.

In this paper we report net entry of each of eighteen amino acids from an equimolar mixture into the marine polychaete, *Pareurythoe californica*. A reduction in rate of entry for a representative of each of the major classes of amino acids is observed when rates are measured in the presence of an equimolar concentration of a representative of any of the other major classes (polar and nonpolar neutral, dicarboxylic, polybasic). Levels of free amino acids found in the natural habitat of the animals are then reported. Net entry is measured using mixtures of amino acids similar in composition and concentration to those found naturally. Finally, rates of net entry from these environmentally meaningful amino acid mixtures are compared with rates of nitrogen excretion and with measurements of aerobic metabolic rate to esti-

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Abbreviations used: DOM (dissolved organic material), HPLC (high performance liquid chromatography), ASW (artificial seawater), OPA (ortho-phthaldialdehyde), DAPI (4,5-diamidino-2-phenylindole), AO (acridine orange).

mate the potential contribution of amino acid uptake to the metabolic requirements of the worms.

MATERIALS AND METHODS

Animals

Pareurythoe californica was collected in the low intertidal area at San Onofre in southern California from the underside of cobbles which lie on a substrate of sand-gravel-rock. The worms were maintained in the laboratory at 23°C in an aquarium containing artificial seawater (ASW) prepared according to Cavanaugh (1964). The animals were fed once a week ("Seafood"; Instant Ocean Hatcheries). ASW was changed every second day. Animals were used within six weeks of collection.

Errant polychaetes such as *Pareurythoe* are opportunistic predators and scavengers, and under natural conditions they presumably feed frequently. As noted above, they were maintained in the laboratory by feeding once a week. Excess food was removed from the aquarium after each feeding. Under these conditions the worms may have starved periodically, which could have led to changes in rates of oxygen consumption, ammonia production, or amino acid uptake. Analysis of our data indicates no effect of our feeding regime on any of these parameters.

Environmental samples

Samples were taken of both the open water washing freely over the cobbles and of the water from the microhabitat of the worms under the cobbles. The latter samples were collected by inserting narrow-gauge tubing between and under undisturbed cobbles and drawing water slowly into a sterile 50 ml syringe. All water samples were filtered immediately in the field after collection, first to remove large particulates (0.45 μm; Millepore Type HA) and then to remove bacteria (0.2 μm; Nuclepore). The filtered samples were placed in sterile culture tubes and stored frozen for later analysis by HPLC.

The water samples were filtered under very gentle pressures. Neither the pressures generated nor the shear forces developed at the surface of the filter were great enough to damage any bacteria present. We have added bacteria to sterile ASW and filtered it, and see no disruption of the bacteria.

HPLC

HPLC methods similar to those employed by Jones and Gilligan (1983) were used to determine the levels of amino acids in the environmental samples, to measure rates of amino acid uptake and ammonia production in laboratory experiments, and to measure free amino acid pools in the organisms. Briefly, 800 μl of filtered sample, diluted if necessary with HPLC-grade distilled water, was combined with 200 μl of ortho-phthaldialdehyde (OPA) reagent and mixed vigorously. The resulting fluorescent amino acid derivatives were separated on a Beckman HPLC system. 500 μl aliquots of derivatized amino acid solution were injected onto an HPLC column assembly consisting of a guard column (70 mm long × 2.1 mm ID) packed with CO:PELL ODS (30 μm particle size; Whatman) followed by an Ultrasphere ODS analytical column (15 cm long × 4.6 mm ID; 5 μm particle size). Derivatives were eluted using gradient profiles composed of 0.05 *M* sodium acetate (pH 6.8), methanol, and small quantities of tetrahydrofuran as an organic modifier to improve separation of glycine and threonine. Peaks were detected and quantified using a Shimadzu C-E1B integrator. Actual concentrations of amino acids were determined by comparison with chromatographically pure standard mixtures. Standards were evaluated approximately every seventh run.

Uptake experiments

Net flux of amino acids was determined as follows. For each trial, one or two worms (about 70 mg wet wt) were washed repeatedly with sterile ASW. After washing, they were placed in a sterile beaker containing a solution of each of the amino acids to be tested at an initial concentration of 200 n*M* made up in 20 ml of autoclaved ASW. In each case a control group was set up without exogenous amino acid to monitor any possible leakage from the worms.

The beakers containing the worms were stirred at 50 rpm on an orbital shaker. This procedure provided moderate mixing without causing observable behavioral disturbances. At timed intervals, 2 ml samples were removed from the medium and immediately filtered through sterile filters (0.2 μm; Nuclepore) and frozen for subsequent analysis by HPLC. Rates of uptake were calculated based on the assumption of exponential depletion of amino acids from the medium. (This assumption was subsequently verified by least squares curve fitting on curves similar to those presented in Fig. 2.) The rate of uptake for each amino acid was calculated from the following expression:

$$K = (\ln [S_0] - \ln [S_t])/t$$

where K is the first order depletion coefficient, S_o is the substrate concentration at the start of the experiment, and S_t is the substrate concentration at time t. The depletion coefficient along with the volume of the medium and the concentration of each amino acid was used to calculate rates of uptake which are expressed as nmoles (gm wet wt)$^{-1}$ h^{-1}.

The rate of net exchange of individual amino acids, determined by HPLC, was compared with the rate of influx of the same ^{14}C-labeled amino acid in selected cases. These experiments were conducted as described above except that single amino acids were used and 0.5 μCi of uniformly labeled ^{14}C-amino acid was added to the initial incubation medium. At each sampling period, a 0.5 ml aliquot of the filtered sample was placed in a scintillation vial and acidified with 100 μl of 0.5 *N* HCl to volatilize any $^{14}CO_2$ formed during the course of the experiment. At least twelve hours after acidification, 4.5 ml of Aquasol II (New England Nuclear) was added to each vial. Twenty-four hours later the samples were counted on a Beckman CPM-100 scintillation counter.

Kinetics

The kinetics of influx were determined for three individual amino acids by determining the initial rates of uptake of ^{14}C-labeled amino acid from starting concentrations ranging from 300 n*M* to 500 μ*M*. Duplicate 0.5 ml samples were removed after 0, 3, 7, 10, and 15 minutes of incubation and prepared for scintillation counting as described above. Uptake rates were calculated by least squares regression of amino acid disappearance from the medium *versus* time. These rates were plotted *versus* their corresponding substrate concentrations and the kinetic parameters $J_{i(max)}$ (the maximal rate of influx) and K_t (concentration at which influx is half-maximal) were determined directly by non-linear least squares regression analysis (Duggleby, 1981).

Internal amino acid pools

To determine amino acid levels in the body wall of *Pareurythoe,* worms were washed in sterile ASW and a piece of body wall was dissected from the midsection.

The tissue was briefly rinsed in ASW, blotted, weighed on an analytical balance, dried to a constant weight at 90°C and reweighed. The dried tissue was rehydrated in HPLC-grade 80% ethanol and homogenized in a Dounce homogenizer. The homogenized tissue was allowed to extract for 24 hours at 4°C. The homogenate was then centrifuged at 2100 × *g* for 10 min. Aliquots of the supernatant were diluted with HPLC-grade distilled water and amino acid levels determined by HPLC.

Bacteria

Three separate procedures were used to evaluate the potential contribution of microorganisms to the observed uptake of amino acids by *Pareurythoe.*

(1) In conjunction with net flux experiments described above, a control group was set up to serve as a check for uptake due to epifauna loosely associated with the surface of the worms. Worms were placed on the orbital shaker in beakers of sterile ASW with no added substrate for 90 min. At this point, the animals were removed and sufficient amino acid stock solution was added to the medium to provide each of the 18 amino acids at a concentration of 200 n*M*. Disappearance of these added amino acids was followed by HPLC analysis of filtered medium samples taken over an additional 90-min period.

(2) Washed, intact animals were frozen with dry ice onto slides coated with gelatin-chrom alum. The frozen worms were embedded in Tissue-Tek (Miles Labs) at −27°C and 10 μm sections cut on a cryostat. The cut sections were placed on a cold slide, thaw-mounted and allowed to air-dry. The sections were stained for 1 min with either 4,5-diamidino-2-phenylindole (DAPI) at a final stain concentration of 0.01 μg ml^{-1}, or acridine orange (AO) at a final stain concentration of 10 μg ml^{-1}, then rinsed in McIlvaines buffer (pH 7.2). The sections were examined on a Zeiss standard microscope at 800× and 1000× with Neo-Fluor objectives. Epifluorescence was produced with a 100 W HBO mercury bulb and either a G 436 exciter filter with an FT 510 dichromatic beam splitter and an LP 515 Barrier filter (AO fluorescence) or a BP 365/10 exciter filter with an FT 390 chromatic beam splitter and an LP 395 barrier filter (DAPI fluorescence). As a check on the mounting and staining procedures, parallel samples of bacteria in detrital material collected from a lab aquarium were processed by the same procedures.

(3) Washed, intact animals were placed in a sterile culture tube and rinsed in a strong stream of ASW. The tube containing the animal was then vortexed for 90 s. The worm was removed and the ASW was treated by procedures similar to those of Daley and Hobbie (1975), Coleman (1980), and Porter and Feig (1980). The sample was fixed by addition of 6% formalin in ASW which had been freshly filtered (0.2 μm; Nuclepore). The sample was then filtered onto 0.2 μm Nuclepore filters which had been stained in Irgalan black (2 g l^{-1} plus 20 ml of acetic acid). The filters were rinsed with distilled water and counterstained with either DAPI or AO. The wet filters were mounted on slides coated with low fluorescence oil and coverslipped with oil. They were then examined by epifluorescence microscopy as described above. Parallel samples washed from detrital material were processed as controls.

Oxygen consumption

The oxygen consumption of *Pareurythoe* in the presence or absence of exogenous amino acids was measured using a Gilson differential respirometer. The reaction flask contained two animals in sterile ASW. After equilibration at 23°C for 30 min, sufficient amino acid stock solution to produce a 3 μM solution or an equivalent volume

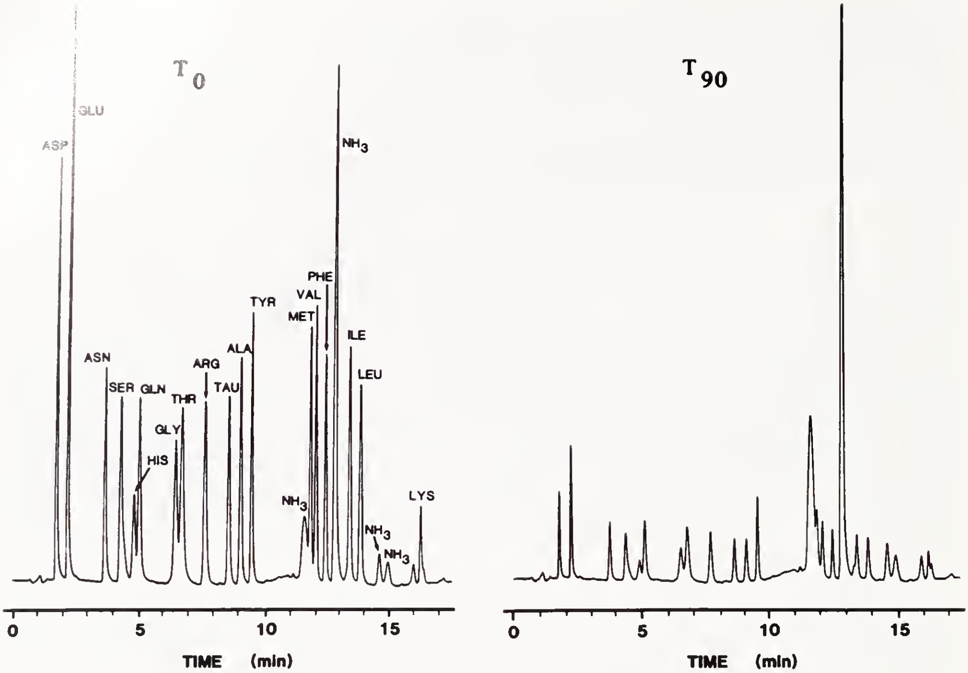

FIGURE 1. Net removal of 18 amino acids by *Pareurythoe.* The chromatogram at the left reflects the composition of the medium at time 0. The initial concentration of each amino acid in this medium was 200 n*M.* The chromatogram at the right shows the medium 90 min after addition of two worms (68 mg wet wt).

of sterile ASW was tipped into the reaction flask and oxygen consumption was measured for the next two hours.

RESULTS

The results shown in Figure 1 are typical of those obtained in six sets of observations measuring net entry of amino acids into *Pareurythoe* from the medium. The chromatogram labeled T_0 represents initial conditions where each of the 18 amino acids was present at a concentration of 200 n*M.* A significant proportion of all amino acids tested was removed during the 90 min incubation period as indicated in the chromatogram labeled T_{90}. This chromatogram also shows that ammonia was liberated over the course of the observations. Quantification of the areas of the peaks in these chromatograms yielded the average rates of uptake given in Table I. Analysis of variance showed that there was no significant difference in the rates of uptake among the polar neutral, nonpolar neutral, acidic, and basic amino acids tested ($P > 0.10$). The average total rate of uptake from our equimolar mixture (total concentration 3.6 μM) was 267.9 ± 18.5 nmoles (g wet weight)$^{-1}$ h^{-1}. The average ammonia production under these conditions was 547.6 ± 36.7 nmoles (g wet weight)$^{-1}$ h^{-1}.

Animals placed in sterile ASW without exogenous amino acids did not lose detectable amounts of any amino acid over a 90 min incubation period, but they did release ammonia at an average rate of 504.7 ± 43.1 nmoles (g wet weight)$^{-1}$ h^{-1}. This rate of ammonia release does not differ significantly from that of the experimental group above ($P > 0.10$). Given the volumes and tissue weights used in these experiments

TABLE I

Average rates of net uptake of 18 amino acids as determined by HPLC

Nonpolar neutral		Acidic	
ALA	16.87 ± 1.03	ASP	18.88 ± 0.71
ILE	15.64 ± 0.72	GLU	17.65 ± 0.73
LEU	15.49 ± 1.40		
MET	17.02 ± 1.60		
PHE	16.23 ± 1.01		
VAL	16.28 ± 0.56		
Polar neutral		**Basic**	
ASN	13.85 ± 0.74	ARG	14.80 ± 0.76
GLY	16.41 ± 1.24	HIS	12.42 ± 0.98
GLN	13.86 ± 1.88	LYS	10.79 ± 1.70
SER	16.20 ± 1.46		
THR	15.00 ± 1.31		
TYR	14.50 ± 0.71		

All values are expressed as nmoles (g wet wt)$^{-1}$ h^{-1} ± SEM. n = 6.

and the sensitivity of our HPLC detector, we could have easily quantified rates of amino acid loss as low as 1.2 nmoles (g wet weight)$^{-1}$ h^{-1}.

Entry of amino acids into *Pareurythoe* undoubtedly occurs at or via the external epithelium. Previous studies on adult marine annelids have shown that non-feeding animals do not drink (Ahearn and Gømme, 1975). It seems to be a general characteristic of uptake of amino acids in adult marine invertebrates that little or no entry occurs via the gut (Stephens, 1972).

To assess the potential contribution of epifaunal contaminants to the observed uptake of amino acids in the preceding experiments, we made one set of observations to measure the uptake by loosely attached epifauna and two separate sets of direct bacterial counts using epifluorescence microscopy. No epifaunal contamination was detected by these procedures although bacteria were clearly visible on the detrital samples processed as controls for the microscopy. Overall, our data support the view that *Pareurythoe* is the agent responsible for uptake, and that bacteria play a negligible role in the process.

Although we do not suggest that the surface of *Pareurythoe* is sterile, the low levels of bacteria that could have escaped our procedures could not provide a significant contribution to the amino acid uptake observed. These results are striking, but not wholly unexpected. Animals that produce surface mucus coats such as polychaete annelids, commonly secrete bacteriocidal or bacteriostatic compounds in the mucus (Rheinheimer, 1975). Also, scanning electron micrographs of *Nereis* prepared specifically to preserve epifauna show no visible contamination (Sieburth, 1965).

To estimate the concentration gradients against which the uptake of amino acids proceeds, amino acid levels in the body wall of *Pareurythoe* were determined by HPLC. The results of these analyses are presented in Table II. These concentrations represent minimum estimates of the intracellular amino acid concentrations in the transporting epithelium since they are based on the total water content of the tissue rather than its intracellular water content. Gradients (internal:external) calculated on the basis of these internal amino acids levels and the initial concentrations present in the test mixture of 18 amino acids ranged from 2×10^4 for histidine to 5×10^5 for asparagine. These are minimum values both because of the probable underestimate of actual intracellular concentrations noted above and because all of the amino acids

TABLE II

Concentrations of amino acids in the body wall of Pareurythoe

Amino acid	Conc. (m*M*)	Amino acid	Conc. (m*M*)
ASN	102.41	ARG	14.05
ALA	31.58	VAL	13.56
ASP	31.02	ILE	13.42
GLU	27.37	GLN	13.38
LYS	19.59	THR	10.78
LEU	18.95	PHE	9.68
MET	17.55	TYR	9.04
SER	15.41	TAU	5.63
GLY	14.86	HIS	4.37

Total amino acid concentration = 372.66 m*M*.
n = 3.

supplied in the medium were reduced substantially below their initial concentration of 200 n*M* during the observations.

To clarify the relationship between influx and the net entry of individual amino acids, simultaneous measurements of influx (disappearance of ^{14}C-labeled amino acid from the medium) and net flux (decrease in concentration of the same amino acid as measured by HPLC) were performed. Such measurements were made for representative acidic (aspartate), basic (lysine), nonpolar neutral (alanine, glutamine), and polar neutral amino acids (serine, glycine). The initial concentration in the incubation medium (^{14}C-labeled plus unlabeled) ranged from 300 to 400 n*M* depending on the specific activity of labeled compounds. Fluxes were measured for 90 min. The results shown in Figure 2 for serine are typical. In no case did least squares regression analysis of the influx and net flux data yield curves whose coefficients were significantly different.

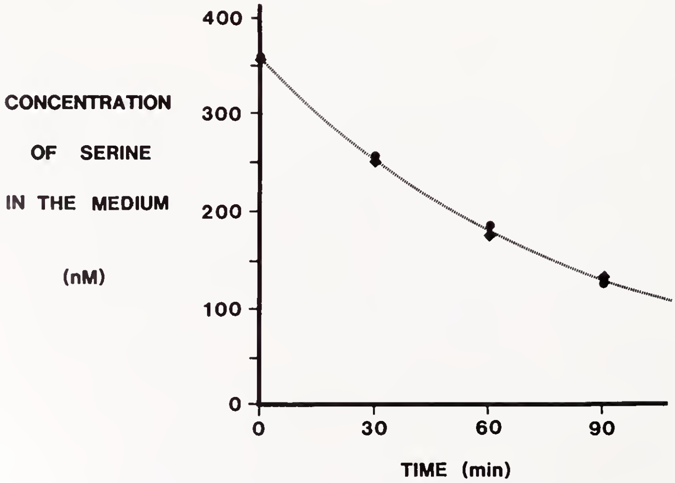

FIGURE 2. Influx of labeled serine compared to net serine flux as measured by HPLC. Influx data are represented by diamonds (♦). Net flux data are represented by circles (●). The exponential curve was fit to both data sets by least squares regression ($r^2 = 0.9975$).

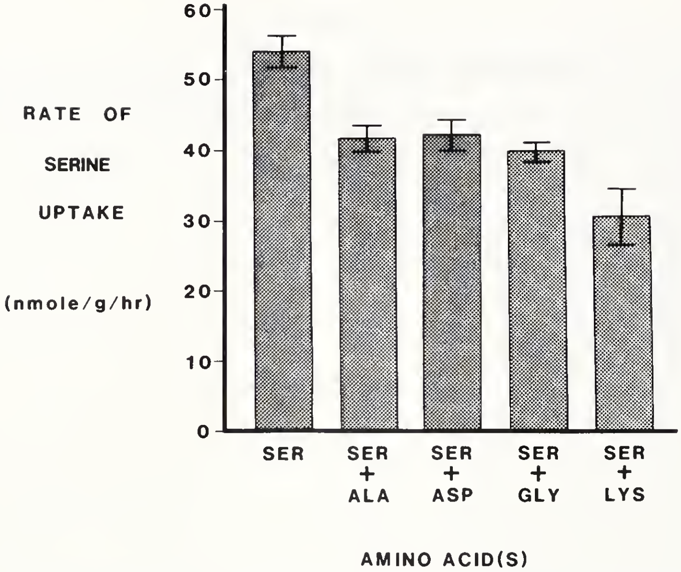

FIGURE 3. Inhibition of serine uptake by equimolar amounts of amino acids from each of the major amino acid classes. Each amino acid was present in the medium at an initial concentration of 200 n*M*. Net flux of each of the amino acids was determined by HPLC analysis of samples of the medium.

The kinetics of influx were investigated for serine, glycine, and alanine. The relationships between the rates of influx for each of these amino acids and their concentrations in the external medium were well described by the Michaelis-Menten equation. Values for $J_{i(max)}$ and K_t were determined directly from Michaelis curves by nonlinear least squares regression analysis. $J_{i(max)}$ values obtained were 3.6 (serine), 4.3 (glycine), and 6.1 (alanine) expressed as μmoles (g wet weight)$^{-1}$ h^{-1}. The corresponding values for K_t (μM) were 29.5 (serine), 36.9 (glycine), and 66.3 (alanine).

It was noted in the above observations that entry rates for these amino acids when presented singly were higher than those in experiments using our test mixture of 18 amino acids. We therefore examined possible interactions between amino acids. Uptake of representatives from each major class was measured when each amino acid was presented alone and when it was paired with an equimolar amount (200 n*M* each) of an amino acid from each class. An exhaustive set of pairings was constructed using serine, glycine, alanine, aspartate, and lysine as substrates. All of the amino acid pairings tested resulted in a 20–40% inhibition of uptake compared to the rate observed when the amino acid was presented alone. The results presented for serine (Fig. 3) are typical.

HPLC analysis of water samples taken at the collection site at San Onofre produced data summarized in Table III. Total amino acids present in the water washing freely over the cobbles ranged from 115 to 350 n*M;* from 8 to 17 different amino acids were identified in the nine samples analyzed. Each of the five water samples taken from under the cobbles contained all 18 amino acids we measured with total concentrations ranging from 3.6 to 17.5 μM. Table III presents the mean value of samples measured. The microhabitat of *Pareurythoe* was richer in both diversity and concentration than the surrounding open seawater. Six amino acids were consistently more abundant in the samples with serine, glycine, alanine, glutamate, aspartate, and lysine accounting for more than 80% of the total present.

TABLE III

Average amino acid concentrations at San Onofre

Amino acid	Microhabitat water	Open water
ASP	652 nM	16 nM
GLU	261	11
ASN	118	3
SER	3129	39
HIS	120	8
GLN	685	7
GLY	2062	36
THR	533	6
ARG	96	—
TAU	57	—
ALA	944	16
TYR	208	—
MET	134	7
VAL	276	5
PHE	144	—
ILE	261	—
LEU	232	—

Microhabitat water n = 5; open water n = 9.

As noted above, interactions among amino acids affected their rates of influx. Therefore, rates of uptake from an amino acid mixture simulating observed substrate composition in the natural habitat were determined and used to make more realistic estimates of entry under natural conditions. The mixture employed contained the six most abundant amino acids from the microhabitat samples present in proportion to their abundance in these samples. The total amino acid concentration was 3.0 μM (*i.e.*, slightly less than the minimum total concentration measured in the microhabitat of the worms at the collection site). Average rates of amino acid uptake from this mixture are listed in Table IV.

The aerobic metabolic rate of *Pareurythoe* was measured in ASW containing either serine, glycine, alanine (at a concentration of 7 μM) or without added substrate. Analysis of variance showed the presence of exogenous amino acids caused no significant change in oxygen consumption. The overall average oxygen consumption was 198 ± 13 μl (g wet weight)$^{-1}$ h^{-1}. Based on the rates of uptake of the six major constituents listed in Table IV, this corresponds to the oxidation of approximately 3.2 μmoles of these amino acids.

DISCUSSION

We have used HPLC analysis to demonstrate net entry of each of an equimolar mixture of 18 amino acids each supplied at an initial concentration of 200 nM (total concentration 3.6 μM). For six individual amino acids chosen to represent the four major classes of amino acids, influx as measured by the disappearance of ^{14}C-labeled substrate equaled rate of net entry as measured by direct chemical quantification via HPLC. These observations exclude the possibility of accounting for the observed influx of labeled amino acids by postulating exchange diffusion (Johannes *et al.*, 1969).

Animals incubated for 90 min in ASW did not lose detectable amounts of any amino acid though they did continue to produce ammonia at rates comparable to those shown by animals exposed to the test amino acid mixture. This observation is

TABLE IV

Amino acid composition of artificial seawater solutions designed to mimic the natural microhabitat of Pareurythoe *and rates of uptake from these solutions*

Amino acid	Concentration (n*M*)	Rates of amino acid uptake [nmoles (g wet wt)$^{-1}$ h^{-1}]
SER	1050	123.49 + 2.67
GLY	750	97.89 + 5.74
ALA	300	37.57 + 0.67
ASP	300	32.19 + 0.26
GLN	300	39.29 + 0.29
LYS	300	17.96 + 5.23
Total	3000	348.39 + 2.48

n = 5.

in agreement with the bulk of observations in the literature. Leakage of amino acids from marine invertebrates can be induced by previous exposure of the animals to high concentrations of substrate (*e.g.*, Jorgensen, 1980; Davis and Stephens, 1984a), but has not been convincingly demonstrated except in cases involving special experimental procedures. Transepidermal leakage has been postulated by Gømme (1982) and by Wright and Secomb (1984). However, this proposed leakage is accompanied by localized active resorption such that net loss to the bulk medium is not measurable.

Rates of uptake by *Pareurythoe* are comparable for all 18 of the amino acids examined. This is unusual among annelids which have been studied. Stephens (1975) reported that *Nereis diversicolor* takes up neutral amino acids more rapidly than acidic amino acids and Jorgensen (1979) reported a similar situation for *N. virens.* Other marine invertebrates vary in the extent to which they discriminate among classes of amino acids. The bivalve, *Mytilus edulis,* exhibits rather catholic tastes (Manahan *et al.*, 1982) while echinoderm larvae strongly prefer neutral amino acids (Davis and Stephens, 1984a, b).

The rate of uptake of amino acids by *Pareurythoe* is relatively rapid compared to that of other animals collected from the same microhabitat at San Onofre (*i.e.*, under the cobbles). Uptake from the test solution of 18 amino acids by *Pareurythoe* was 268 nmoles (g wet weight)$^{-1}$ h^{-1}. Under identical conditions, the apodus sea cucumber, *Leptosynapta albicans,* removed 16 of the 18 amino acids. The total uptake was 97 nmoles (g wet weight)$^{-1}$ h^{-1}, neutral amino acids were removed much more rapidly than acidic amino acids and basic amino acids were not taken up at measurable rates. We were unable to demonstrate net entry of any amino acids in the flatworm, *Notoplana acticola.* In previous work, Davis *et al.* (1985) examined uptake of 15 amino acids, each at an initial concentration of 125 n*M*, by the brittle star *Ophionereis annulata* collected at the same location. Recalculating from their data to the higher concentration used in the present study produces a rate of 67 nmoles (g wet weight)$^{-1}$ h^{-1}.

The kinetic parameters reported here for *Pareurythoe* fall well within the range of those reported for epidermal amino acid transport for other marine annelids (see Stephens, 1972; Wright and Stephens, 1978). However, kinetic data such as these should be interpreted cautiously. The presence of unstirred layers at epithelial surfaces is now realized to have important consequences for the interpretation of kinetic data for transport in vertebrate intestine (Winne, 1973; Thomson and Dietschy, 1980). This has been shown to apply to transport by invertebrate epithelia as well (Wright *et al.*, 1980). The fact that the epidermal surfaces of marine polychaetes show a significant unstirred layer is understandable in terms of their architecture. In addition to the surface mucus coat, the epidermis of annelids has a protein-polysaccharide cuticle. Gømme (1982) presented evidence that the cuticle reduces the mobility of small molecules by two to three orders of magnitude compared to the bulk aqueous medium. This would enhance the discrepancy between observed bulk kinetic parameters and the actual behavior of the transporters in the epithelium.

As reported in Results, the uptake of representatives of all of the major classes of amino acids was influenced by the presence of equimolar amounts of other amino acids, even at very low concentrations. We have not attempted to explore the details of kinetics of such interactions. However, the results are reported here because they are obviously germane to estimation of net influx under natural conditions.

Water samples taken from the site at which *Pareurythoe* was collected indicate that the microhabitat of the worms was rich in free amino acids compared to the open water washing over the cobbles under which they are found. Total concentration in the microhabitat was roughly 10 μM while the open water averaged about 100 n*M*. This range of concentrations agrees with previous studies of amino acid levels in comparable environments (Stephens, 1972; Crowe *et al.*, 1977; Henrichs and Farrington, 1979; Davis *et al.*, 1985). The high degree of spatial and temporal variability in levels of amino acids in both habitats make the ranges rather broad.

Estimates of the potential contribution of exogenous amino acids to the nutritional and metabolic requirements of *Pareurythoe* were made by correlating rates of uptake from mixtures consisting of the six amino acids most prominent in the habitat of the worms with rates of oxygen consumption. The mixture employed contained the amino acids in proportion to their observed natural abundance with a total concentration of 3 μM, *i.e.*, slightly below the minimum total concentration measured in the field. At this concentration, amino acid uptake could account for roughly 10% of the reduced carbon required to sustain oxidative metabolism. Extrapolation to the maximum total concentration measured in samples from the microhabitat (about 18 μM) suggests that as much as 50% of the carbon required for oxidative metabolism could be supplied by uptake.

These figures are based on comparison with the measured rate of oxygen consumption and make the further assumption that the amino acids acquired by this pathway are fully oxidized. They are not intended to be interpreted as statements concerning the actual fate of exogenous amino acids acquired from the environment. Neither are they intended to defend the position that the metabolism of these worms is exclusively aerobic in character. With respect to the fate of exogenous amino acids, we know from numerous studies in the literature as well as observations on *Pareurythoe* not cited here that they are incorporated into various compounds in the organisms as well as persisting in the internal free amino acid pool for considerable periods of time. The comparison is designed to call attention to the fact that the input of external amino acids via this pathway presumably spares provision of reduced carbon by other pathways which would be required to sustain carbon balance and provide for growth in the absence of such uptake from the environment.

Similarly, the comparison of the amino nitrogen provided by amino acid uptake with the rate of ammonia excretion is not intended to imply that the amino acids are promptly deaminated on entry and constitute the proximate source of ammonia. Again, it is drawn to indicate that entry of amino nitrogen by this pathway spares other potential nutritional sources and/or utilization of internal reserves present in the animals.

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CYPHONAUTES' CILIARY SIEVE BREAKS A BIOLOGICAL RULE OF INFERENCE

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ABSTRACT

Biologists routinely assume that a mechanism demonstrated in one organism will be found in others in which form, function, ancestry, and physical parameters are similar. These criteria failed to predict the feeding mechanism of the cyphonautes larva of bryozoans. Larval and adult bryozoans feed with cilia similar in size and distribution, with similar current velocities, and similar Reynolds numbers. Nevertheless, the larvae filter particles with a sieve of stationary cilia whereas the adults respond to individual particles with an induced local reversal of ciliary beat and concentrate particles without filtration. The larva's ciliary sieve is doubly unexpected because it requires a ciliary stiffness at the maximum recorded for cilia.

INTRODUCTION

Extrapolation of conclusions from one organism to another is based on morphological and functional similarity, evolutionary relationship, and similarity of size and physical properties. When a mechanism is studied in detail for one species, the same mechanism is routinely assumed to occur in species that meet these criteria of similarity. This basis for generalization about mechanisms rarely fails. These criteria led to the prediction that the cyphonautes larvae of bryozoans would capture particles by the mechanism that has been observed in the adults. The prediction failed.

Larval and adult bryozoans both feed on small suspended particles of food, and both use a similar arrangement of ciliary bands to capture and concentrate the suspended particles. The cilia are arranged in parallel frontal, laterofrontal, and lateral bands (Atkins, 1955; Strathmann, 1973; Winston, 1978) (Fig. 1). The lateral cilia are about 20 μm long and beat with the effective stroke perpendicular to the ciliary bands and away from the frontal surface. The lateral cilia create a feeding current of 1 to 2 mm/s. The laterofrontal cilia are about 20 to 30 μm long and stationary. Observations of particle motion indicate that the frontal cilia beat parallel to the ciliary bands. Food particles are retained upstream from the band of lateral cilia and transported along the frontal surface toward the mouth. This consistency suggests that food particles are concentrated by the same physical mechanism in both larva and adult. Nevertheless, high speed films of particle capture show two different mechanisms.

In the adults the band of lateral cilia concentrates particles by local reversals of beat induced by individual particles (Strathmann, 1982). Because about 50 to 100 μm of ciliary band changes beat to capture a 10 μm particle (Fig. 2), each particle must be captured along with a much larger surrounding parcel of water. The capture and redirection of water along with the particle is inferred from the alteration of ciliary beat because at low Reynolds numbers the alteration of ciliary motion will immediately affect the motion of surrounding water. The adult therefore concen-

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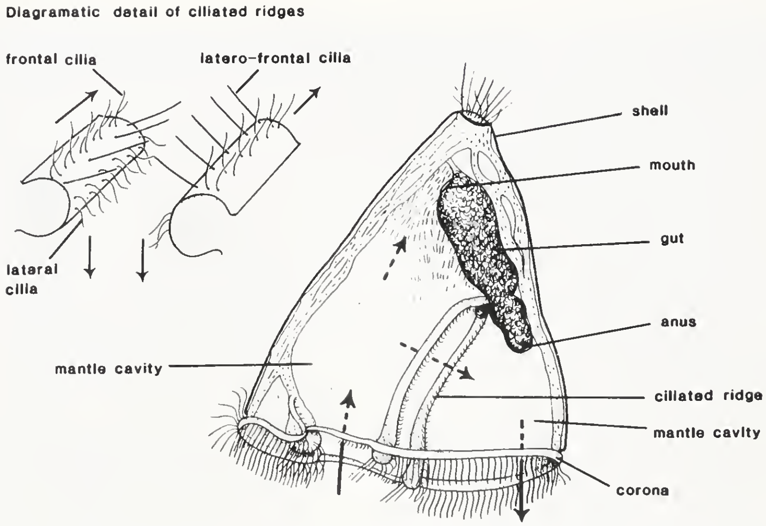


FIGURE 1. Bands of cilia on the two ridges in the mantle cavity of a bryozoan larva are at upper left. Arrows show particle paths near lateral and frontal cilia. Bands of cilia on tentacles of adult zooids are similar. The position of the ciliated ridges and feeding currents in the mantle cavity of a bryozoan larva are at lower right.

trates particles without employing a filter. Instead, particles are redirected to the frontal surface of the tentacle by brief and local alterations of currents by the lateral cilia. The water redirected with a particle is a tiny fraction of the water that passes the tentacles wherever there are no particles to trigger reversals of ciliary beat; therefore the degree of concentration of suspended particles is enormous. For concentration of particles in this system the mechanics of triggering a ciliary reversal is relevant; the mechanics of filtration is not (Strathmann, 1982). The cyphonautes larva was expected to employ the same mechanism (Strathmann, 1973), but high speed films have given no indication of capture by local reversals of ciliary beat and instead demonstrate that the larva uses laterofrontal cilia as a stationary ciliary sieve.

MATERIALS AND METHODS

Larvae were collected from San Juan Channel near the Friday Harbor Laboratories in Washington and from the Gulf Stream off the Smithsonian Marine Station at Link Port, Florida. The larvae from Friday Harbor were filmed at 60 frames per second with a cinecamera equipped with a timing light that marked margins of the 16 mm film. Particles retained on the sieve of laterofrontal cilia were photographed in more transparent bryozoan larvae from the Gulf Stream by electronic flash. The abundance of adults suggests that the larvae from Friday Harbor were *Membranipora membranacea* and the larvae from the Gulf Stream *Membranipora tuberculata.* All observations and photographs were with differential interference contrast optics. The combination of these optics and high speed film results in grainy photos in shades of grey with narrow depth of focus (Figs. 2, 3) but demonstrates events not visible by other means. The films are clearer in motion than in still prints, but even the prints support or reject some hypotheses on particle capture. The cyphonautes larvae in Figure 3 were capturing polystyrene divinylbenzene spheres of 5 μm diameter of the type used to calibrate electronic particle counters. The alga *Dunaliella tertiolecta*

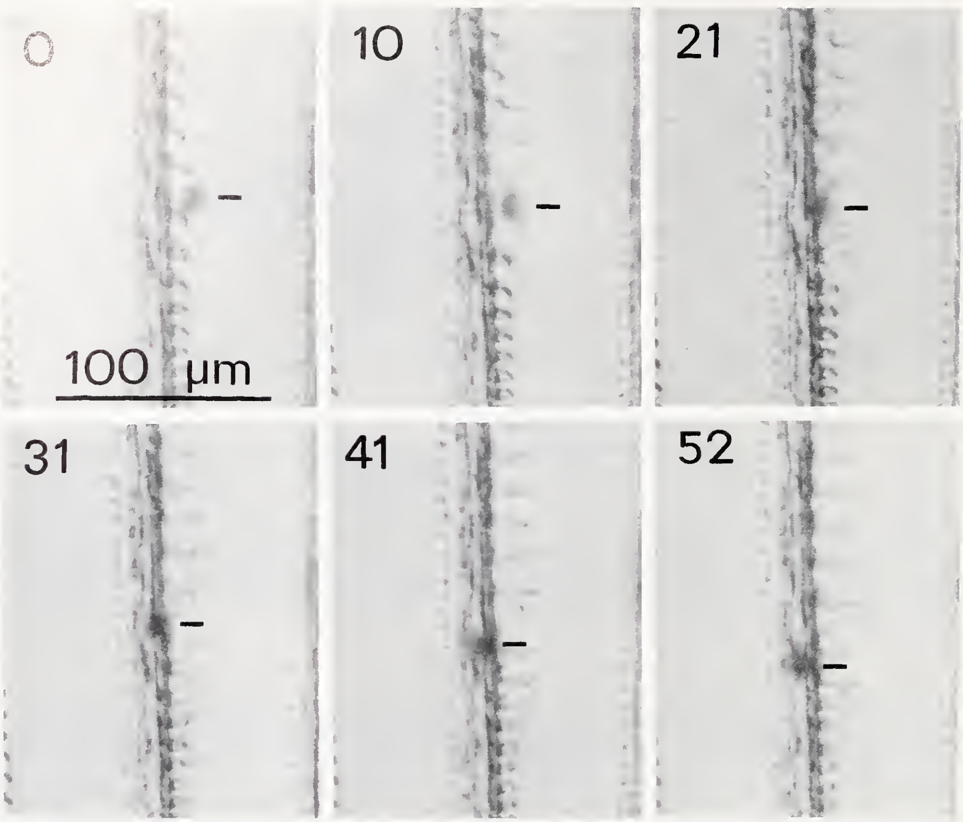


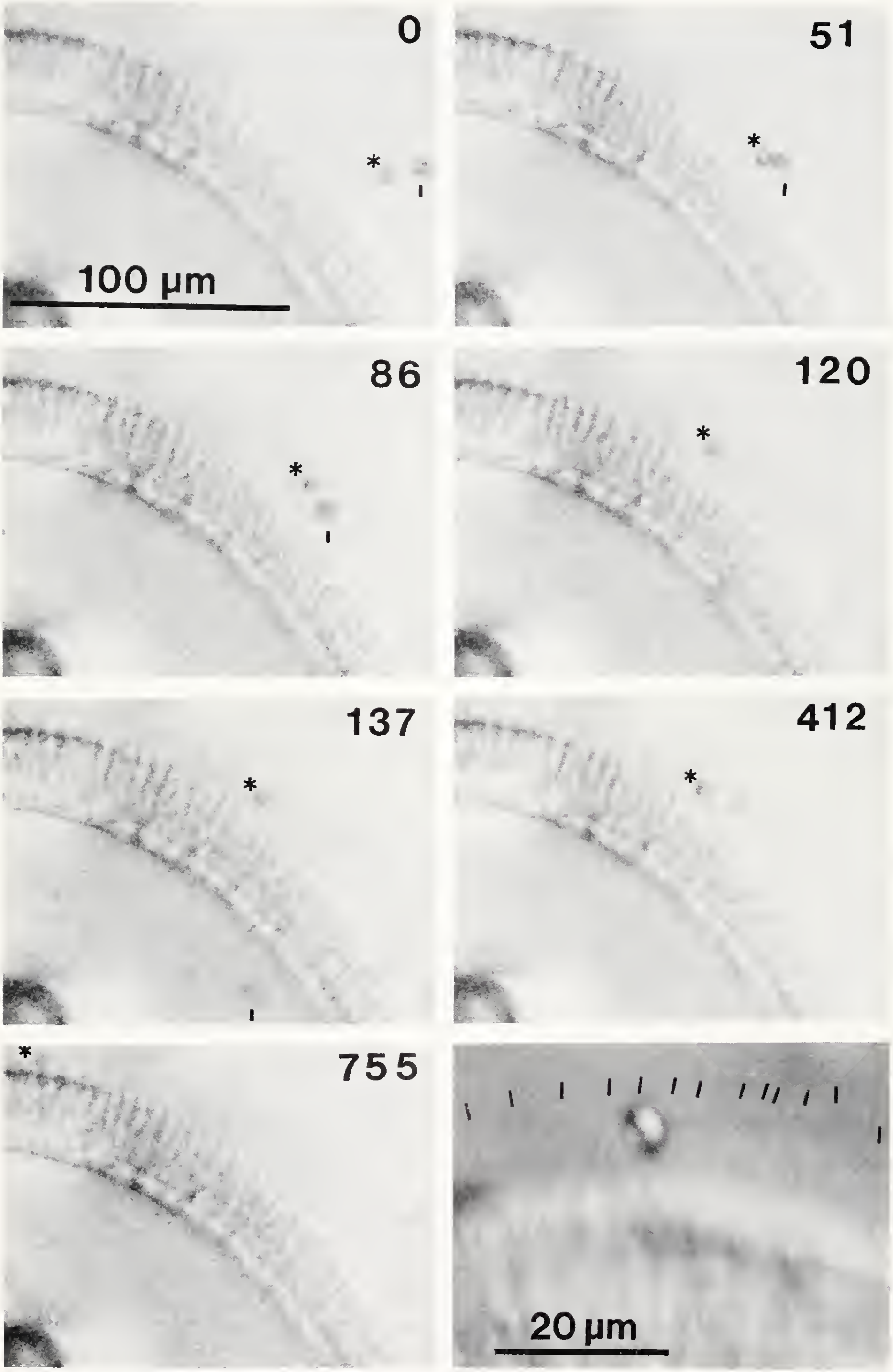
FIGURE 2. Capture of a particle by induced local reversal of lateral cilia on the tentacle of an adult bryozoan. The tentacle is viewed from the frontal surface so that the phased waves of beat of lateral cilia are visible on both sides of the tentacle. The black bar marks the particle's position. Within 10 ms the waves of ciliary beat are disrupted over about 50 μm of the adjacent band as the cilia alter beat in response to the particle. (Note the regularity of metachronal waves and positions of recovery and effective strokes at 0 ms compared to those near the particle at 10 ms.) More cilia alter beat between 21 and 52 ms as the particle is moved toward the frontal surface (center of tentacle) and then proximally along the tentacle (downwards) toward the mouth.

(about 10 μm) was also used in observations of the cyphonautes. The adult bryozoan in Figure 2 was capturing an algal cell of about 10 μm.

RESULTS

In contrast to the adults, bryozoan larvae captured particles by sieving with their band of stationary laterofrontal cilia: the bands of lateral cilia produced feeding cur-

FIGURE 3. Capture of a particle by a sieve of stationary laterofrontal cilia of a larval bryozoan. The ciliated ridge is viewed from the lateral surface. The asterisk marks the captured particle; the vertical bar marks a particle passing through the sieve. The captured particle is caught at the 51 ms frame and remains nearly stationary for 0.35 s while the other particle passes nearby. At 755 ms the particle is being transported along the frontal surface of the ciliary ridge toward the mouth. There is no discernible disruption of beat of the lateral cilia, whose metachronal waves appear as dark and light bands on the cilated ridge. Lower right: 5 μm sphere caught by two stationary laterofrontal cilia; black bars mark visible laterofrontal cilia.


0
100 µm
51
86
120
137
412
755
20 µm

rents as in the adult bryozoans, but the cinefilms indicate that larvae concentrated particles by filtration rather than by an induced local reversal of beat. Each particle was retained upstream without the disruption of metachrony that would indicate a reversal of beat of lateral cilia (Fig. 3). Also, particles remained almost stationary in the upstream position while lateral cilia continued to beat and other particles passed nearby (Fig. 3). Particles lodged against the upstream side of the row of laterofrontal cilia and then remained motionless as long as several seconds while the lateral cilia continued beating. Laterofrontal cilia were spaced more closely than 5 μm apart and retained plastic spheres of 5 μm diameter (Fig. 3). The lack of motion of particles retained by the larva was in striking contrast to the rapid motion of particles retained by the adult (Strathmann, 1973, 1982). Thus particle motion, ciliary motion, and the position of particles on laterofrontal cilia all indicated sieving.

Particles caught on the laterofrontal cilia eventually either moved along the tract of frontal cilia toward the mouth to be eaten or passed the laterofrontal cilia and were lost to the excurrent chamber (Fig. 3). Previously unfed larvae ate almost all particles captured. We could not observe the mechanism by which particles were transferred from the sieving laterofrontal cilia to the upstream cilia that carry food to the mouth.

Could there be a behavioral switch, such that larva or adult could employ both sieving and induced reversal of beat? Comparative observations have failed to support this hypothesis. Motion of captured particles has been observed for at least two species of bryozoan larvae (this study) and four species of bryozoan adults (Strathmann, 1973, 1982), and the observations included both adult and larval stages of a *Membranipora* species from San Juan Channel. R. L. Zimmer (pers. comm.) also observed sieving by the laterofrontal cilia of bryozoan larvae. In all comparisons sieving has been specific to the larval stage, and local reversal of currents has been specific to the adult.

DISCUSSION

Many animals' sieves are composed of parallel cilia or setae, each supported at only one end. If these fibers bend too far under the added drag of a food particle, they will lose the particle. Does the flexural stiffness of cilia place limits on sieving by laterofrontal cilia? Studies of animal filtration have addressed the physics of fluid and particle movement (Strathmann, 1971; Fenchel, 1980; Koehl, 1981; LaBarbera, 1984; Silvester and Sleigh, 1984), but not the mechanical properties of filtering fibers. A simple model provides an estimate of the requisite stiffness. Consider a spherical particle of 5 μm diameter (D) that encounters a stationary cilium at a distance (L) of 20 μm from the base of a cilium with a velocity (U) of 1 mm/s. When the particle is stopped by the stationary laterofrontal cilia, it is exposed to the continuing current from the lateral cilia. The drag force (F) on the particle predicted by Stokes' law, $F = 3\pi \mu UD$, is about 5×10^{-11} N although accuracy of the estimate is reduced by the proximity of surfaces and by accelerations from nearby lateral cilia. The predicted deflection of the cilium at the point of loading is $x = FL^3/3EI$, with EI the flexural stiffness of the cilium (Gordon, 1976). The highest estimate of flexural stiffness for a cilium (Baba, 1972) is 2 to 3×10^{-19} N m^2. This estimate for flexural stiffness implies deflection of about 1 or 2 μm. This small deflection should not prevent sieving, but a greater deflection would release particles from the sieve. Thus the laterofrontal ciliary sieve appears to be operating near the mechanical limits for a ciliary sieve. Baba's (1972) estimate is for abfrontal cilia of *Mytilus edulis*. Other published estimates of ciliary flexural stiffness are lower by more than an order of magnitude (Strathmann *et al.*, 1972; Okuno and Hiramoto, 1979). The flexural stiffness of single cilia may be near the limit required for sieving particles.

Ciliary sieves have been reported for other animals, but in these the cilia are either shorter or combined into thicker compound structures (Fenchel, 1980; Dral, 1967). Stationary laterofrontal cilia may occur throughout a related group of animal phyla; they are reported with varying evidence from larval and adult bryozoans (Atkins, 1955; Winston, 1978), larval (Strathmann, 1973) and adult (Gilmour, 1978) phoronids, and adult brachiopods (Gilmour, 1978), but their function is unknown. For the adult bryozoans, phoronids, and brachiopods that have been carefully examined, the motion of captured particles indicates local reversal of currents by the lateral cilia (Strathmann, 1973, 1982). The same is true for larval enteropneusts and larval echinoderms (though these lack laterofrontal cilia) (Strathmann, 1971; Bonar and Strathmann, 1976), and films of feeding echinoderm larvae confirm that induced ciliary reversal is the mechanism of particle capture (Strathmann *et al.*, 1972). All five phyla belong to a larger related group, the oligomera. Because the induced reversal mechanism is characteristic of five related phyla, a different mechanism in the bryozoan larvae is especially surprising. In retrospect one might argue that the close juxtaposition of laterofrontal cilia across a closed chamber is an arrangement more conducive to sieving than the more separated bands on tentacles of adult bryozoans, but brachiopods also have parallel closely arrayed ciliary bands enclosed by a shell and are not known to employ a sieve (Strathmann, 1973). Indeed, the bryozoan larvae are the only animals known to sieve particles by a palisade of long, separate cilia.

Species are too numerous for the underlying mechanisms in a process to be demonstrated on every stage of development of each one. Some basis is required for generalizing from intensive study of a few. The usual guides for extrapolation are homology, analogy, and similarity of physical parameters. These guides have failed in this instance. Generalizations based on homology, analogy, and physical similarity cannot be abandoned (we have used them throughout this paper in generalizing about differences between larval and adult bryozoa), but because these criteria occasionally fail, the generality of mechanisms demonstrated in a few model systems must remain uncertain.

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